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RT-PCR and Matrix-Assisted Laser Desorption-Ionization Time-of-Flight Mass Spectrometry Diagnostic Target Performance Reflects Circulating Severe Acute Respiratory Syndrome Coronavirus 2 Variant Diversity in New York City

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As severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) continues to circulate, multiple variants of concern have emerged. New variants pose challenges for diagnostic platforms because sequence diversity can alter primer/probe-binding sites (PBSs), causing false-negative results. The Agena MassARRAY SARS-CoV-2 Panel (Agena Bioscience) uses RT-PCR and mass spectrometry to detect five multiplex targets across N and ORF1ab genes. Herein, we use a data set of 256 SARS-CoV-2-positive specimens collected between April 11, 2021, and August 28, 2021, to evaluate target performance with paired sequencing data. During this time frame, two targets in the N gene (N2 and N3) were subject to the greatest sequence diversity. In specimens with N3 dropout, 69% harbored the Alpha-specific A28095U polymorphism that introduces a 3′-mismatch to the N3 forward PBS and increases risk of target dropout relative to specimens with 28095A (relative risk, 20.02; 95% CI, 11.36 to 35.72; P < 0.0001). Furthermore, among specimens with N2 dropout, 90% harbored the Delta-specific G28916U polymorphism that creates a 3′-mismatch to the N2 probe PBS and increases target dropout risk (relative risk, 11.92; 95% CI, 8.17 to 14.06; P < 0.0001). These findings highlight the robust capability of Agena MassARRAY SARS-CoV-2 Panel target results to reveal circulating virus diversity, and they underscore the power of multitarget design to capture variants of concern.

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Since the recognition and global spread of coronavirus disease 2019, nucleic acid amplification testing has been the diagnostic mainstay for identifying new severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infections. Historically, the majority of diagnostic target primers and probes have been designed from viral genomes characterized early in the pandemic. However, in the 2 years since the first SARS-CoV-2 genome was identified, viral variants have emerged in response to various evolutionary pressures. The emergence of variants of concern (VOC) and variants of interest poses a hurdle for diagnostic assays as sequence variation generates mismatches to primer/probe-binding sites (PBSs), potentially causing target dropout and false-negative results. Failure to promptly detect infections with emerging variants that may be associated with greater transmissibility and infectivity represents a substantive risk for forward transmission in the community.

The diagnostic assays that are most robust for detection of SARS-CoV-2 and least affected by viral sequence changes are designed to amplify two or more targets across different viral genes [eg, open reading frame 1 ab (ORF1ab), nucleocapsid (N), spike (S), and envelope (E)]. This redundancy can compensate for individual target dropout due to inherent biochemical differences and analytical sensitivities among targets or due to emergent viral genome diversity at PBS sequences. Several groups have reported the utility of platforms with multiple targets to accurately detect emerging SARS-CoV-2 variants. Furthermore, target performance patterns on these platforms can be used to elucidate dynamics of circulating variants in communities. Indeed, multiple reports have described the use of dropout of diagnostic targets designed at S genes or N genes as a screen for PBS-specific polymorphisms that have arisen among emergent SARS-CoV-2 viruses such as the Alpha variant (B.1.1.7 lineage).

In early 2021, the Delta variant (B.1.617.2 lineage) of SARS-CoV-2 surged in India [CDC COVID Data Tracker, https://covid.cdc.gov/covid-data-tracker/#variant-proportions, last accessed July 4, 2021; The New York Times, https://www.nytimes.com/2021/06/22/health/delta-variant-covid.html (subscription required), last accessed July 4, 2021]). Soon Delta and its sublineages (AY.x) quickly spread across the globe (New York City Department of Health, https://www1.nyc.gov/site/doh/covid/covid-19-data-variants.page, last accessed July 4, 2021; Pango Network, https://www.pango.net/network/new-ay-lineages, last accessed November 29, 2021). By fall 2021, despite increased vaccination rates and infection prevention methods, Delta and its sublineages became predominant in New York City (NYC) and abroad (Centers for Disease Control and Prevention, https://covid.cdc.gov/covid-data-tracker/#variant-propportions, last accessed July 4, 2021; NYC Health, https://www1.nyc.gov/site/doh/covid/covid-19-data-variants.page, last accessed July 4, 2021)). Given that emergent variants may be associated with resistance to therapeutic antibodies, or reduced vaccine effectiveness, diagnosing new infections is vital. Several mutation-specific assays have been designed to detect lineage-specific S gene mutations in Delta and older variants. However, few assays have been evaluated for their ability to capture contemporary variants in routine clinical settings, or to shed light on sequence diversity in virus genes other than spike (S).

We recently reported the power of the Agena MassARRAY RAY SARS-CoV-2 Panel (Agena Bioscience, San Diego, CA) for detection of emergent SARS-CoV-2 variants in NYC during the first months of 2021 and the utility of diagnostic target performance to illuminate sequence diversity among then-predominant Alpha variant genomes. This assay combines RT-PCR and matrix-assisted laser desorption-ionization time-of-flight (RT-PCR/MALDI-TOF) mass spectrometry to detect five targets in the SARS-CoV-2 genome. Coincident with the spread of the Delta VOC in NYC, we observed a distinct change in the target performance patterns that we hypothesized reflected ongoing viral evolution, prompting us to examine the corresponding genomic sequence data.

For the current study, we compared paired RT-PCR/MALDI-TOF diagnostic target data and genome sequencing data for SARS-CoV-2—positive specimens that were collected between April 11, 2021, and August 28, 2021, and recovered, deidentified, and sequenced as part of the Mount Sinai Health System (MSHS) Pathogen Surveillance Program at the Icahn School of Medicine at Mount Sinai. Herein, a data set of 256 genomes from SARS-CoV-2—positive clinical specimens was used to highlight the viral genomic diversity at each PBS on the Agena MassARRAY SARS-CoV-2 Panel. Moreover, we specifically identify PBS mismatches in the N gene and lineage-specific sequence diversity that affected diagnostic performance patterns for specimens collected over the course of ongoing viral evolution in NYC.

Materials and Methods

Ethics Statement

This study was reviewed and approved by the Institutional Review Board of the Icahn School of Medicine at Mount Sinai (HS#13-00981).

SARS-CoV-2 Specimen Collection and Testing

Upper respiratory tract (eg, nasopharyngeal, anterior nares) and saliva specimens collected between April 11, 2021, and August 28, 2021, for SARS-CoV-2 diagnostic testing in the Molecular Microbiology Laboratories of the MSHS Clinical Laboratory (which is certified under Clinical Laboratory Improvement Amendments of 1988, 42 U.S.C. §263a, and meets requirements to perform high-complexity tests) were eligible for inclusion in this study. Overall, 177,059 upper respiratory tract and saliva specimens underwent SARS-
CoV-2 clinical testing at MSHS Molecular Microbiology Laboratories during the study period, and a subset of 256 were deidentified and underwent SARS-CoV-2 next-generation sequencing as previously described.24–26 For this retrospective study, deidentified data were used for diagnostic specimens tested on the Agena MassARRAY SARS-CoV-2 Panel and MassARRAY System (CPM384) platform. Details of processing and SARS-CoV-2 testing of upper respiratory tract and saliva specimens have been described previously.24,27

This RT-PCR/MALDI-TOF assay detects five viral targets: three in the nucleocapsid (N) gene (N1, N2, and N3) and two in the ORF1ab gene (ORF1A and ORF1AB). Results were interpreted as positive if ≥2 targets were detected or negative if <2 targets were detected. If no MS2 internal extraction control and no targets were detected, the result was invalid and required repeat testing of the specimen before reporting. If internal extraction control was detected and no targets were detected, the sample was interpreted as negative.

SARS-CoV-2 Sequencing, Assembly, and Phylogenetic Analyses

SARS-CoV-2 viral RNA underwent RT-PCR amplification and next-generation sequencing followed by genome assembly and lineage assignment using a phylogenetic-based nomenclature as described by Rambaut et al25 using the Pangolin version 3.1.14 tool and PANGO-version 1.2.81 nomenclature scheme (GitHub, https://github.com/cov-lineages/pangolin, last accessed April 15, 2022) as previously described.25,26 Ultimately, this yielded 244 complete genomes (≥95% completeness) and 12 partial genomes (<95% completeness). Complete viral genome sequences were deposited in the publicly available Global Initiative on Sharing Avian Influenza Data database (Global Initiative on Sharing Avian Influenza Data, www.gisaid.org, last accessed April 15, 2022); accession identifiers are indicated in Supplemental Table S1.

Agena Target Sequence Alignment

Agena MassARRAY target detection results were matched to the corresponding genome sequences. Primer and probe sequences for each Agena target were obtained from published US Food and Drug Administration Emergency Use Authorization documentation for the Agena MassARRAY SARS-CoV-2 Panel (MassARRAY SARS-CoV-2 panel instructions for use, Agena Bioscience, Inc.) (Table 1). We generated reverse-complement sequences for reverse primers for all five targets and probes that are designed in the reverse orientation (eg, N1-N3). An unaligned FASTA file including sequence data for the clinical specimens and the Wuhan-Hu-1 reference sequence was generated for each of the 15 primers/probes. The data are available at the National Center for Biotechnology Information nucleotide database (NC_045512.2) and GenBank (MN908947.3) (https://www.ncbi.nlm.nih.gov/nuccore). Alignment of specimen sequences with each primer/probe sequence was performed by using the Multiple Alignment using Fast Fourier Transform platform29,30 as previously described.34 To enable inclusion of incomplete genomes that had intact regions sequenced at PBVs, uninformative sequences (eg, with ambiguous letters) were not removed. Otherwise, the default settings were used to align all sequences to the reference genome, which generated a resulting FASTA alignment file for each primer and probe sequence.

Sequence Variation in Primer/Probe Target Regions

To examine sequence diversity at each primer/probe PBS region, nucleotide diversity per bp was calculated by using the PopGenome population genomics analysis package in R (version 4.1.2).31 Nucleotide diversity per site (π) was calculated by dividing the nucleotide diversity of all sequences within each PBS sliding window by the number of nucleotide sites in each corresponding PBS. To identify and calculate mismatch frequency at each PBS, custom Unix-code (Zenodo, AceM1188/SACOV_primer-probe_analyses, https://doi.org/10.5281/zenodo.4920818, last accessed June 9, 2021) was used to identify mismatches at each nucleotide position within each primer and probe sequence as described previously.24 PBS mismatch frequency by position of forward/reverse primer and probe sequences were calculated on Microsoft Excel version 16.54 (Microsoft Corporation, Redmond, WA). To account for differences in completeness of consensus genomes, the number of PBS mismatches was normalized to the number of nucleotides in the PBS of each specimen consensus sequence.

To compare global sequence data for lineages and polymorphisms relevant to this study, we queried publicly available sequence data from the Global Initiative on Sharing Avian Influenza Data EpiCoV database through their online user interface (Freunde von GISAID e.V., https://www.epicov.org, last accessed October 16, 2021). Using available filters for collection date ranges, amino acid substitutions, and variant classification, we calculated the frequencies of distinct genomes deposited. Specifically, to distinguish nucleotide polymorphisms at SARS-CoV-2 position 28,916, the number of variations at the corresponding 215th amino acid in the N protein was identified. Nucleotide changes were determined by comparing the codon of the native glycine amino acid (28916-GGU-28918) versus the codons of the variant amino acids. These changes (nucleotide change underlined) included cysteine (UGU), serine (AGU), and arginine (CGU) amino acids.
For statistical comparison of the fraction of PBSs with mismatches in genomes with detected targets versus those with dropout, normality was assessed by using the D’Agostino-Pearson test (GraphPad Prism version 9.2.0, GraphPad Software, San Diego, CA). All distributions were nonparametric, and thus a Mann-Whitney test (two-tailed) was performed (GraphPad Software). To determine if specific mismatches were associated with specific target dropout results, specimens were grouped according to the presence or absence of the mismatch of interest (in the setting of no other mismatches) and detection or dropout of the target of interest; this approach resulted in a 2 × 2 contingency table that underwent association testing by using Fisher’s exact test. In addition to P value, relative risk ratios and 95% CIs are provided for association testing.

Statistical Analysis

Diagnostic target PBSs, genome sequences were processed by using a custom Unix-code (Zenodo, https://doi.org/10.5281/zenodo.3775031, last accessed June 9, 2021).

Data Availability

SARS-CoV-2 sequencing read data for all study genomes were deposited in the Global Initiative on Sharing Avian Influenza Data database (www.gisaid.org); accessions are indicated in Supplemental Table S1.

Results

Of 448 SARS-CoV-2—positive specimens, 117 (26%) had all five targets detected; the remaining specimens had one (n = 247) or more (n = 84) target dropout. For the subset of 256 SARS-CoV-2—positive specimens sequenced in our study, all five diagnostic targets were detected in 96 (38%), with the remaining having one (n = 140) or more (n = 20) target dropout (Supplemental Table S1).

When we calculated weekly detection rates for individual targets among these SARS-CoV-2—positive specimens, the N2 (0.61) and N3 (0.77) targets had the lowest average detection rates (Figure 1A). Notably, N2 and N3 detection rates were lowest at distinct periods of time. Among weeks with ≥3 SARS-CoV-2—positive specimens sequenced, the N2 detection rate was lowest (0.13) in the week ending July 31, 2021, and the N3 detection rate was lowest (0.41) in the week ending May 1, 2021. We hypothesized that this shift was due to changes in the population of circulating SARS-CoV-2 variants. When individual target detection rates were compared versus the reported prevalence of variants in NYC during the same time period, the N3 detection rate was lowest when the Alpha variant (B.1.1.7 + Q.x) was

### Table 1: Agena MassARRAY RT-PCR/MALDI-TOF Target Primer and Probe Sequences

| Target | Primer/probe | Sequence | SARS-CoV-2 coordinates (NC_045,512.2*) |
|--------|--------------|----------|--------------------------------------|
| N1     | For          | 5'-AGACGCGACATCATGGTGGTGTTG-3' | 28,654–28,673 |
| N1     | Rev          | 5'-TCGAGAGGTTGTAGCCAGGTTTTG-3' | 28,761–28,740 |
| N2     | For          | 5'-GGGAGTCTTTCTCCTGCTAGAAT-3' | 28,881–28,902 |
| N2     | Rev          | 5'-CATACCTAGCTGCCTCAAGTGT-3' | 28,979–28,958 |
| N2     | Probe        | 5'-GCAAGGCAAGACGACATCACC-3' | 28,937–28,916 |
| N3     | For          | 5'-GTTGATAGCTGGTCTGCTTCAA-3' | 28,096–28,077 |
| N3     | Rev          | 5'-ACTACAAGACTACCCAATT-3' | 28,192–28,173 |
| N3     | Probe        | 5'-GAAACTGTTGATTTAATCTCCGATA-3' | 28,138–28,118 |
| ORF1A  | For          | 5'-AATTACAGTATCATGACATCGATA-3' | 3232–3244 |
| ORF1A  | Rev          | 5'-CAATAGCTCTGACATCCTGTT-3' | 3335–3314 |
| ORF1A  | Probe        | 5'-GTTCAACCTCATTAGAAT-3' | 3286–3302 |
| ORF1AB | For          | 5'-CCTGTTGCCGGTTTACACTTAAA-3' | 13,342–13,362 |
| ORF1AB | Rev          | 5'-ACAGTCTTGGGACATCGCTG-3' | 13,460–13,442 |
| ORF1AB | Probe        | 5'-ATCAACCTCCGAGACCC-3' | 13,432–13,416 |

*https://www.ncbi.nlm.nih.gov/nuccore.

For, forward; MALDI-TOF, matrix-assisted laser desorption-ionization time-of-flight; Rev, reverse.
A  

Figure 1  RT-PCR/matrix-assisted laser desorption-ionization time-of-flight target detection rate in severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)–positive specimens. A: Heatmap depicting the proportion of SARS-CoV-2–positive specimens that have detectable RT-PCR/matrix-assisted laser desorption-ionization time-of-flight targets (N1, N2, N3, ORF1A, and ORF1AB) according to week from April 11, 2021, through August 28, 2021. The total number of SARS-CoV-2–positive specimens and the number of SARS-CoV-2–positive specimens sequenced according to pathogen surveillance are depicted above each week (column). Gray boxes indicate weeks in which no specimens were positive for SARS-CoV-2 on this platform. B: Stacked area plots depicting frequencies of SARS-CoV-2 variants reported by publicly available New York City (NYC) Department of Health surveillance data within the same time frame.

Impact of Mismatches on Target Performance

We aligned each diagnostic target PBS to each of the 256 SARS-CoV-2 genome sequences and calculated the number of mismatches to each target PBS for both detected and undetected targets (Figure 2). For four targets (N1, N3, ORF1A, and ORF1AB), when the individual target was detected, there was perfect complementarity (0 mismatches) between genome sequences and forward, reverse, and probe sequences for almost all (>99%) specimens. However, for specimens in which the N2 target was detected, mismatches occurred both at N2 forward (34.3%) and reverse (46.6%) PBSs (Figure 2B). Specifically, among specimens with detectable N2 target, one to three mismatches to the forward PBS and up to one mismatch to the reverse PBS were observed. Notably, specimens with N2 target dropout had a significantly greater number of mismatches to the N2 probe PBS (Figure 2B). The same was found for specimens with N3 dropout and mismatches to the N3 forward PBS (Figure 2C). Of interest, more mismatches to the N2 reverse PBS were found for specimens in which the N2 target was detected than for those with N2 dropout. Indeed, the majority of specimens with detectable N2 target (85%) harbored mismatches in N2 forward or reverse PBSs, but 0% of specimens with detectable N2 target harbored mismatches to the N2 probe PBS.
Site-specific Polymorphisms Affect Target Performance

Because mismatch position in PBSs can affect primer/probe binding to target sites, we characterized mismatch frequencies according to position in each PBS (Figure 2). At least one mismatch was identified in every target PBS with the exception of the N1 reverse, N3 reverse, and the ORF1AB forward primers, which had zero mismatches to all possible haplotypes in our data set. To assess the level of sequence diversity across each target, the nucleotide diversity per site (π) was calculated for each PBS sequence. Overall, genomes sequenced in this time frame exhibited the most diversity at N2 target PBSs (≥0.01260) followed by the N3 forward PBS (0.01075) (Figure 3, B and C). Diversity at these targets was at least sevenfold more than that at all other target PBSs. Notably, this diversity corresponded with a predominance of Delta (56.7%), Alpha (15.7%), and Iota (16.5%) variant genomes in this data set.

To identify individual mismatches associated with target dropout, we measured the proportion of genomes with a mismatch at each PBS position and compared this level across specimens that yielded target detection or dropout (Supplemental Figure S1). From 5'-to-3' direction, there were mismatches to the first to third bp of the N2 forward primer PBS (SARS-CoV-2 genome positions 28,881 to 28,883) in genomes that yielded both N2 target detection and dropout (Figure 3B, Supplemental Figure S1B). Overall, there were 192 specimens with at least one mismatch to the first 3 bp of the N2 forward primer. Of these, 49 (26%) had a concurrent trio of substitutions in the N gene: G28881A, G28882A, and G28883C. The remaining 143 (74%) harbored a single G28881U substitution. The GGG-

![Figure 2](image-url)
Figure 3  Alignment of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) haplotypes to RT-PCR/matrix-assisted laser desorption-ionization time-of-flight targets. Multiple sequence alignment of haplotype sequences to forward, reverse, and probe binding sites for N1 (A), N2 (B), N3 (C), ORF1A (D), and ORF1AB (E) targets. In each alignment, the reference sequence (NC_045,512.2; https://www.ncbi.nlm.nih.gov/nucleotide) is annotated above, comprising color-coded nucleotides. The coordinates of the reference sequence are annotated at the bottom corners of each alignment. Each row represents a haplotype sequence that aligns to the target site. Absolute counts of each haplotype (eg, Hap_1, Hap_2, and so forth) within the data set and counts
to-AAC and the G28881U substitutions were independent of one another, and no combinations of the two were detected. The majority of specimens with the trio (88%) had a detectable N2 target. In contrast, 78% of specimens with G28881U resulted in dropout, which, despite its position at the 5′-end of the N2 forward primer, represents a significant association of G28881U with N2 target dropout ($P < 0.0001$).

Interestingly, of 123 genomes with N2 target dropout, 111 (90%) were identified that harbored a mismatch at the terminal 3′ base of the 22-bp-long N2 probe (Figure 3B, Supplemental Figure S1B). All mismatches are the result of a guanine-to-uracil substitution in the N gene (G28916U). This substitution was not identified among any of the 131 specimens with detectable N2 target. In fact, the relative risk of N2 target dropout in the setting of the G28916U substitution was 11.9 times greater than genomes with the native guanine (95% CI, 8.17 to 14.06; $P < 0.0001$). We also determined that all 111 genomes with the G28916U substitution have the G28881U substitution. The linkage of the G28881U and G28916U polymorphisms likely explains why the former (a 5′-end mismatch to the N2 forward PBS) is associated with N2 target dropout. Indeed, 100% of genomes ($n = 32$) that possess the G28881U substitution alone resulted in N2 target detection.

In the current study, 25 (69%) of 36 specimens with N3 target dropout had a mismatch at the penultimate position of the 3′-end of the 20-bp-long N3 forward primer (Figure 3C, Supplemental Figure S1C). This mismatch corresponded with the adenine-to-uracil substitution in the ORF8 gene (A28095U), which causes a 20-fold risk of N3 target dropout relative to genomes with the native nucleotide (95% CI, 11.36 to 35.72; $P < 0.0001$). This outcome is consistent with our findings earlier in the pandemic, before the current study period.

We assessed whether the associations between mismatch and corresponding target dropout persist when quantity of virus genomic material in specimens is controlled as previously described. When analyses are limited to specimens for which all non-N2 targets are detected, the association of the G28916U substitution with N2 dropout remains significant (relative risk, 20.2; 95% CI, 14.58 to 47.48; $P < 0.0001$). In addition, when analyses are limited to specimens for which all non-N3 targets are detected, the A28095U substitution remains significantly associated with N3 target dropout (relative risk, 31.03; 95% CI, 10.95 to 91.10; $P < 0.0001$). These results indicate that the G28916U and A28095U substitutions are associated with N2 and N3 target dropout, respectively, independent of quantity of SARS-CoV-2.

### Lineage-specific Variation and Target Dropout

To determine whether target dropout was the result of lineage-specific variation, we examined phylogenetic lineages of genomes harboring the distinct polymorphisms. Among 25 specimens with N3 target dropout in this study, 23 (92%) belonged to the B.1.1.7 lineage (Alpha variant) and two were partial genomes whose phylogenies could not be determined. These specimens were collected from April 11, 2021 (PV36791), through June 30, 2021 (PV37690), which mirrored the prevalence of the Alpha variant in NYC (Figure 1B).

We next examined the lineage of genomes harboring the G28916U substitution (Figure 4). Of 111 specimens that bore the G28916U polymorphism and resulted in N2 target dropout, the earliest was from July 1, 2021 (PV37692), and the latest from August 21, 2021 (PV38720). All genomes corresponded with lineages and sublineages of the Delta variant. Specifically, these included the B.1.617.2 ($n = 67$) lineage and AY.20 ($n = 2$), AY.23 ($n = 1$), AY.25 ($n = 22$), AY.3 ($n = 17$), AY.33 ($n = 1$), and AY.5 ($n = 1$) sublineages. We did not find the G28916U substitution among any of the other variant lineages in our data set (Figure 4A).

We broadened our analyses to interrogate SARS-CoV-2 genomes deposited globally for the G28916U polymorphism. We used the publicly available EpiCoV (Global Initiative on Sharing Avian Influenza Data) database and queried 2,103,844 sequences collected during the same time frame of this study (Figure 4B). The Delta variant (B.1.617.2 + AY.x) was found to be predominant in the deposited genomes, comprising 62% of all genomes ($n = 1,313,492$). The majority of these Delta genomes (82%) harbored the G28916U substitution. Of the remaining Delta genome sequences, almost all (99.3%) had the native guanine nucleotide, with a minority of sequences (<0.1%) harboring G28916A and G28916C substitutions. The G28916U polymorphism, which results in the G215C amino acid substitution in the N protein, is almost exclusive to Delta variant genomes but was rarely (<0.02%) reported in other variant groups, including Alpha (B.1.1.7 + Q.x), Beta (B.1.351 + B.1.351.2 + B.1.351.3), Gamma (P.1 + P.1.x), Lambda (C.37 + C.37.1), or Mu (B.1.621 + B.1.621.1).

### Discussion

The current study presents a robust assessment of the impact of the emergence of SARS-CoV-2 variants on detection of individual target genes included in the Agena MassARRAY SARS-CoV-2 Panel. We focused on a 5-month period...
characterized by rapid changes in circulating VOC and variants of interest.

Molecular diagnostic tests to detect SARS-CoV-2, first designed almost 2 years ago, are challenged by continued viral circulation and rapid viral diversification, with continual emergence of new lineages. At the same time, recognition that some virus variants can exhibit increased infectivity and disease severity among a broader population that now includes younger individuals, has raised awareness regarding assay sensitivity and risks for missed diagnoses. Several studies have reported the impacts of viral sequence variation on conventional diagnostic platforms or have highlighted, in *silico*, potential interference of variant polymorphisms with publicly available target sequences. However, there are limited reports on assay performance in *actu* for detection of the Delta VOC and sublineages, and other recent VOC/variants of interest, including Omicron (BA.1 and BA.2), which, most recently, arose from South Africa in late 2021 (World Health Organization, https://www.who.int/news/item/26-11-2021-classification-of-omicron-(b.1.1.529)-sars-cov-2-variant-of-concern, last accessed March 3, 2022). Notably, preliminary, unpublished in *silico* analyses of global Omicron genomes, including those captured at MSHS, reveal a high level of sequence conservation at PBSs with rare mismatches and low risk for dropout. However, this finding remains to be further analyzed as new sublineages emerge.

We used publicly available primer/probe sequences of the Agena MassARRAY SARS-CoV-2 Panel and a deidentified data set from clinical specimens that included individual target detection data and whole-genome sequencing. The goal was to generate a snapshot of virus sequence diversity at each target PBS and to determine the extent to which polymorphisms are associated with individual target performance. Interestingly, we found that distinct viral substitutions resulted in N2 and N3 target dropout. Consistent with what we previously reported, the A28095U substitution is a B.1.1.7-associated polymorphism that introduces a mismatch at the 3’-end of the N3 forward PBS that results in N3 target dropout. Notably, a marked change in the pattern of target dropout occurred during the course of the study with the introduction of the Delta VOC; we found that the G28916U polymorphism unique to a subset of Delta variants results in a 3’-mismatch to the N2 probe and is associated with N2 dropout. Our findings highlight the robust capability of this multiple-target platform to capture diverse viral variants, as well as the ability of target performance to reflect lineage-specific sequence variation outside the conventional S gene.

![Image](https://example.com/image.png)

**Figure 4** Delta-specific substitution interferes with N2 diagnostic target detection. A: Stacked bar plots depict the composition of severe acute respiratory syndrome coronavirus 2 variants in 254 Mount Sinai Health System (MSHS) genomes tested by RT-PCR/matrix-assisted laser desorption-ionization time-of-flight. Bar plots reflect absolute number of genomes with N2 target dropout (left) and those with N2 target detection (right). Variant groups are color-coded, and genomes with the G28916U polymorphism are depicted by a hatched pattern. B: Stacked bar plot of publicly available sequences (Global Initiative on Sharing Avian Influenza Data [GISAID]; see Materials and Methods) from the same time frame of this study depicting absolute numbers of variant genomes and presence or absence of the G28916U polymorphism. Color-coding and patterns are the same as in panel A.
variant-defining polymorphisms may not persist over continued circulation of SARS-CoV-2 and, thus, new target formulations will need to be continuously considered. As most efforts to accommodate new variants have been to redesign primers and probes or include more targets in this region, this may become impractical as inclusion of additional reactions can become costly and introduce more room for error. Furthermore, although multiplexing assays allow for single-pot reactions, the level of target redundancy is limited by the optical detection system as more fluorophores complicate conventional diagnostic methods. Therefore, as the coronavirus disease 2019 pandemic continues and new variants emerge, there can be great utility in existing assay platforms that target multiple regions outside the $S$ gene.

Our findings also highlight the level of sequence diversity observed at each of these five target loci among sequences from VOC/variants of interest detected through August 2021. Among the Agena MassARRAY SARS-CoV-2 Panel, $N$ gene targets (eg, N2, N3) are challenged by the greatest level of nucleotide diversity, which can result in dropout of each. Consistent with phylogenetic analyses of global sequences, the N2 target region has the greatest sequence diversity. Although a number of polymorphisms in the $N$ gene are under positive selection, the evolutionary pressures exerted on A28095U (ORF8/K68*) and G28916U (N2/G215C) polymorphisms have not exhibited positive selection or purported epistatic interactions. This is likely due to the earlier time frames for which each of these phylogenetic studies were performed (eg, December 2020 through May 2021) and the lack of robust functional genomic studies in SARS-CoV-2. Thus, the fate of each of these polymorphisms is unclear among emergent VOC, and each should be taken into consideration for use of this diagnostic platform and others that target the same $N$ gene regions going forward.

As SARS-CoV-2 variants continue to arise, robust platforms with high target redundancy for virus detection and diagnosis are vital to capture new or existing lineages. As contemporary polymorphisms undergo selection, evaluation of diagnostic target performance in the clinical setting can help elucidate ongoing viral dynamics in patient communities and highlight platform target combinations that should be further optimized. Lastly, our results reinforce the need for continuing surveillance of assay target result patterns to increase the sensitivity of current molecular tests for SARS-CoV-2 detection and to assure the adequate diagnosis of coronavirus disease 2019 worldwide.

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Supplemental Data

Supplemental material for this article can be found at http://doi.org/10.1016/j.jmoldx.2022.04.003.

Author Contributions

M.M.H., R.B., P.S., A.E.P.-M., M.R.G., M.D.N., and E.M.S. provided clinical samples for the study. M.M.H., R.B., B.G., P.S., L.C., F.C., H.S., A.H., M.S.P.S.P.S.G., and A.E.P.-M. accessioned clinical samples. A.S.-G., A.v.d.G., M.S.P.S.P.S.G., and H.v.B. performed next-generation sequencing experiments. R.S. provided next-generation sequencing services. A.S.-R. performed genome assembly, data curation, and genotyping. M.M.H. performed alignments and mismatch analyses. M.M.H., R.B., J.D.R., M.R.G., M.D.N., T.E.S., V.S., H.v.B., E.M.S., and A.E.P.-M. analyzed, interpreted, or discussed data. M.M.H., E.M.S., and A.E.P.-M. wrote the manuscript. M.M.H., E.M.S., and A.E.P.-M. conceived the study. E.M.S. and A.E.P.-M. supervised the study. H.v.B., V.S., A.E.P.-M., and E.M.S. raised financial support. M.M.H. and A.E.P. are the guarantors of this work and, as such, had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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**Supplemental Figure S1** Positions of primer/probe-binding site (PBS) mismatches and target detection results. Line graphs depict the percentage of specimen genomes with mismatches at individual positions across the N1 (A), N2 (B), N3 (C), ORF1A (D), and ORF1AB (E) target PBSs. Forward (For), reverse (Rev), and probe PBSs are depicted in separate plots for each target. For each PBS, two line plots depict mismatches in genomes from specimens that yielded a detected target result (magenta) or target dropout (turquoise). The percentage represents the number of genomes with mismatches at each position relative to the number of genome sequences detected or not detected by each target (annotated in each graph).