Title: Multiplex Target-Redundant RT-LAMP for Robust Detection of SARS-CoV-2 Using Fluorescent Universal Displacement Probes

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Abstract

The increasing prevalence of variant lineages during the COVID-19 pandemic has the potential to disrupt molecular diagnostics due to mismatches between primers and variant templates. Point-of-care molecular diagnostics, which often lack the complete functionality of their high throughput laboratory counterparts, are particularly susceptible to this type of disruption, which can result in false negative results. To address this challenge, we have developed a robust Loop Mediated Isothermal Amplification assay with single tube multiplexed multi-target redundancy and an internal amplification control. A convenient and cost-effective target specific fluorescence detection system allows amplifications to be grouped by signal using adaptable probes for pooled reporting of SARS-COV-2 target amplifications or differentiation of the Internal Amplification Control. Over the course of the pandemic, primer coverage of viral lineages by the three redundant sub-assays has varied from assay to assay as they have diverged from the Wuhan-Hu-1 isolate sequence, but aggregate coverage has remained high for all variant sequences analyzed, with a minimum of 97.4% (Variant of Interest: Eta). In three instances (Delta, Gamma, Eta), a high frequency mismatch with one of the three sub-assays was observed, but overall coverage remained high due to multi-target redundancy. When challenged with extracted human samples the multiplexed assay showed 100% sensitivity for samples containing greater than 30 copies of viral RNA per reaction, and 100% specificity. These results are further evidence that conventional laboratory methodologies can be leveraged at the point-of-care for robust performance and diagnostic stability over time.

Introduction

The COVID-19 pandemic is an unprecedented crisis in the modern era, spreading across the planet in a matter of months, infecting and killing millions, while disrupting the lives of billions (1). An essential element of the response strategies to COVID-19 is diagnostic testing, which informs clinical intervention, quarantine, and epidemiological monitoring (2). Nucleic acid amplification tests (NAATs)
remains the most accurate approach for diagnosis of infectious diseases including SARS-CoV-2 infection. However, RNA viruses like SARS-CoV-2 have a high mutational rate, which can result in elevated levels of sequence diversity accumulating as they propagate. This is a critical obstacle for NAATs because mismatches between the primer oligonucleotides and the template sequences can impair an assay and produce false negative results. As transmission has progressed, SARS-CoV-2 has diversified in distinct lineages, each with signature mutations throughout the genome (3). The emergence of this genetic diversity has rendered some NAATs susceptible to false negative results, causing these tests to be altered or withdrawn by the U.S. FDA (4). This challenge posed by mutation for NAATs is not limited to SARS-CoV-2; similar phenomena have been observed for other human pathogens (5, 6).

Laboratory testing strategies to lessen this risk include redundant testing with alternative methods, diagnostic panels with multiple target regions (7), and/or primer sets with degenerate bases to account for known genetic variability (8). While degenerate primers are accessible and inexpensive, they are often limited by assay design constraints and do not account for unknown or novel mutations. Repeat and multiple testing is an effective strategy, but requires additional resources, labor, and complexity of design or implementation. These considerations are manageable in contemporary diagnostic laboratories but can be prohibitive in lower resource settings. Nearly all laboratory assays for SARS-CoV-2 use redundant targets to mitigate mutations and an internal control to account for sample processing or interference.

A critical aspect of the Center for Disease Control and Prevention’s (CDC) Strategy for Global Response to COVID-19 (2020-2023) is augmenting our current ability to rapidly identify COVID-19 infections so that the chain of transmission can be disrupted. Essential to this effort is the development of diagnostics that can be performed at the point-of-care (POC); that minimize the time to result (TTR) of the test and are deployable in otherwise underserved populations. These settings are inherently “low resource,” and necessitate diagnostic methods with simplified chemistry, hardware, and limited sample processing relative to the standard of practice for molecular diagnostics, polymerase chain reaction (PCR). Advancements in isothermal nucleic acid amplification technologies over the past three decades largely satisfy these constraints while still providing high sensitivity. This has led to a boom in isothermal amplification technologies and NAATs based on them (9). Despite their advantages, there are some areas where the isothermal NAATs are lacking when compared to PCR. Single-pot multiplexing has been infrequently demonstrated despite being a prerequisite for internal amplification control (IAC) systems and useful for multiple target redundancy (10–12) In this work we look to contribute to this capability as it relates to Loop Mediated Isothermal Amplification (LAMP) for the detection of SARS-COV-2.

Herein we describe a multiplexed reverse transcriptase LAMP (mRT-LAMP) combining three assays, each targeting a unique region of the nucleocapsid (NC) gene, and an IAC assay to validate diagnostic viability with a negative result. To accomplish this, we have designed a universal target specific fluorescence probe system. In this method, engineered adapter sequences are incorporated into the LAMP amplicons which then serve as a template for detection by displacement probes. The resulting assay chemistry is sensitive, specific, and durable while simplifying the development process. We evaluate the limit of detection, cross-reactivity with other organisms, and reactivity with extracted RNA from patients infected SARS-CoV-2.

**Materials and Methods**

**Preparation of TFpol polymerase**

Plasmid preparation and protein expression and purification were performed as previously described (13).
Primer and IAC design

Three sets of LAMP primers (Table 1) targeting different regions of the SARS-CoV-2 nucleocapsid phosphoprotein were designed manually using the primer design feature of Geneious 8.1.9 (14) against the SARS-CoV-2 reference sequence (GenBank accession number: NC_045512). IDT OligoAnalyzer (15) and NUPACK (16) were used to evaluate designs in silico. Each target design consists of the six conventional LAMP primers: F3, B3, FIP, BIP; LF, and LR (17). The IAC was designed using a composite primer technique (18) for LAMP. IAC template sequence was derived from target region “NC1” by substituting the target loop primer binding sites with engineered sequences. One of the engineered IAC loop sites was used as an IAC loop primer while the other was omitted, so that the IAC assay uses a single loop primer (LFc mut in Fig. 1B). For each primer set a loop primer was modified by the addition of an engineered probe adapter sequence at its 5’ end, with all targets sharing a common adapter and the control assay using a second unique adapter sequence. Primer oligonucleotides were sourced from Integrated DNA Technologies (Coralville, Iowa, USA).

Table 1: Primer, Probe, and control sequences for the SARS-CoV-2 mRT-LAMP. For Primers and probes, F2/B2 sequences are underlined, non-template linker sequences are italicized, and adapter sequences are shown in bold.

| SARS-CoV-2 NC1 Primers | Sequence                                                                 |
|------------------------|-------------------------------------------------------------------------|
| NC1 FIP                | CCACTGCGTTCCTCCATTCCTTTCCCCGCATTACGTGTTTGGT                              |
| NC1 BIP                | GGATCAAAAAACACGTGGTTTCCATGTTGAGGACGCC                                      |
| NC1 LF                 | TGTTACTGCGGACATTGGAATCT                                                  |
| NC1 LB + Target adapter| ACCAACACCTCACATCACACACATAATAAGTTTAGCAATACATGCGTCTTG                     |
| NC1 F3                | TGACCACAAATCAGGGCA                                                      |
| NC1 B3                | ATCTGGACTGCTATTGGGTGTTA                                                  |

| SARS-CoV-2 NC2 Primers | Sequence                                                                 |
|------------------------|-------------------------------------------------------------------------|
| NC2 FIP                | CAGCTTTGCGCCAGTCTCTGTTGAGGACGTCATACATGCTTTACATGCGTCTTG                  |
| NC2 BIP                | CCTCCCTATGGGTGCTAAAAGTCAACTTGATCTCTTTGGGTATGACAGTTCA                   |
| NC2 LF                 | GTAGTAAATACATGCTGGACT                                                   |
| NC2 LB + Target adapter| ACCAACACCTCACATCACACACACATAATAATATGGGTGGTCAACTGAGGGAG                  |
| NC2 F3                | CTACTACGAGAGCATACG                                                   |
| NC2 B3                | GCAGCATTGCTAGGAGATT                                                  |

| SARS-CoV-2 NC3 Primers | Sequence                                                                 |
|------------------------|-------------------------------------------------------------------------|
| NC3 FIP                | CACCTTCCGCTCCGATGCTCTGTTGTGACGTTGAAATAG                                 |
| NC3 BIP                | CTTCCTATGGGTGCTAAAAGTCAAATCTGATCTCTTTGGGTATGACAGTTCA                   |
| NC3 LF + Target adapter| ACCAACACCTCACATCACACACACACATAATAATATGGGTGGTCAACTGAGGGAG                  |
| NC3 B3                | GCAGGACCCAATCATGCGTNTTATGACAGGTGCAATATGCTATTCAAG                         |
| IAC (NC1) primer       | Sequence                                                                 |
| IAC FL + Control adapter| ACCACACCTCACATCACACACACACACATAATAACTACATCACCACACACATCAC                  |

| SARS-CoV-2 UDP         | Sequence                                                                 |
|------------------------|-------------------------------------------------------------------------|
| Target (CoV) UDP Probe | FITC-CCATCAGCACCAGCAGACTACCACACCCCATCAGGCACCACCACACACACACACACACACATACAATA |
| Target (CoV) UDP Quencher| TTGGTGCGGAGGTGTTGAGGCTTTGCTGATTGAGG - Iowa Black® FQ                   |
Universal displacement probe design

Two engineered universal displacement probes (UDP) corresponding to the target adapter or IAC adapter sequence were designed. Each UDP consists of an oligonucleotide duplex with a 3’ overhang and a fluorophore quencher pair (19). The adapter sequence is located at the 3’ overhang position, with a fluorophore spacer sequence at the 5’ end and a 5’ terminal fluorophore (6-FAM or TEX615). The quencher (Iowa Black® FQ or Black hole Quencher®-2) sequence is a complementary fluorophore spacer sequence and is labeled with a 3’ dark quencher so that it quenches the fluorophore when annealed. Probe adapters and universal displacement probe sequences were generated from randomized sequence and manually modified in Geneious, using OligoAnalyzer and NUPACK as secondary analysis tools, to minimize dimer and hairpin structures within and between the probes and adapted loop primers. All designs were tested individually and multiplexed in combination against synthetic dsDNA gBlocks™ (Integrated DNA Technologies, Coralville, Iowa, USA) target and ssDNA IAC Ultramer™ (Integrated DNA Technologies, Coralville, Iowa, USA) fragments to inform iterative design changes to individual assays. Final design iterations are reported; probes and quenchers oligonucleotides were sourced from Integrated DNA Technologies (Coralville, Iowa, USA).

Patient samples

A panel of 102 human respiratory specimens was used to evaluate our mLAMP assay performance. These specimens collected from nasal or nasopharyngeal swabs were suspended in 3mL viral transport medium (Becton Dickinson 220220), aliquoted, and stored at −80°C until testing as described (20). The panel was originally characterized by OpenArray (ThermoFisher Scientific, Waltham, MA, USA) to contain at least 30 COVID-POS across a wide range of concentrations and 30 COVID-negative samples as well as other samples identified as positive for other respiratory diseases including, but not limited to, Streptococcus pneumoniae, Influenza, seasonal Coronavirus, Adenovirus, and Enterovirus. Supplementary Table S1 shows detailed profile in each specimen used in this study. Samples were reassessed in house for the presence of SARS-CoV-2 RNA, as described below, to account for losses during freeze-thaws, storage, or extraction. In-house results were used as the reference standard. Specimens were collected and tested for SARS-CoV-2 infection as part of the Seattle Flu Study, as approved by the Institutional Review Board at the University of Washington (IRB#: STUDY0006181). Informed consent was obtained for all participant samples, including for use of de-identified, remnant specimens.

Patient sample preparation
Specimens were extracted using the QIAamp Viral RNA Mini Kit (Qiagen # 52906) according to the manufacturer’s protocol. 100µL of sample was mixed with 40µL negative VTM (to reach the manufacturer’s recommended 140µL input), extracted, and eluted in 70µL buffer. 5µL aliquots were prepared for single use to avoid free thawing and stored at –80°C until use.

**mRTLAMP protocol**

20µL mRT-LAMP reaction contains 5mM DTT, 8 mM magnesium sulfate, 20 mM Tris-HCl, 10 mM ammonium sulfate, 10 mM KCl, 0.5% (v/v) Triton X-100, 1µM of each FIP and BIP primers, 500 nM of each LF and FB primers, 200 nM of each FV and BV primers, 200 nM FAM-tagged UDP probe and TEX 615 UDP probe, 300 nM Quencher 1 and Quencher 2 probes, 10 units of RNasin® Plus Ribonuclease Inhibitor (Promega, N2611), 6 units of WarmStart® RTx (NEB, M0380L), 0.7 µg TFpol polymerase, and 2 units of thermostable inorganic pyrophosphatase (NEB, M0296L). 5µL of extracted RNA was added to 15µL mLAMP reaction mixture and incubated at 63.3°C for 1 hour. Fluorescence measurements for FAM and TEX 615 signal, indicating SARS-CoV-2 and IAC amplification, respectively, were taken every 25 seconds (accounting for 13 second cycle and read times).

**RT-PCR protocol**

The RT-PCR protocol was prepared as previously described (20). Each 20µL RT-PCR reaction contains 5mM DTT, 200µM ea. dNTP, 1x of either N1, N2, or RP primer/probe mix (IDT, 10006770), 80mM Tris-sulfate, 20mM ammonium sulfate, 4mM magnesium sulfate, 5% (v/v) glycerol, 5% (v/v) DMSO, 0.06% (v/v) IGEPAL CA-630, 8.4% (w/v) trehalose, 0.05% (v/v) Tween-20, 0.5% (v/v) Triton X-100, 7.5U reverse transcriptase (NEB M0380L), and 2.5U polymerase (NEB M0481L). 5µL of extracted RNA was added to the 15µL RT-PCR reaction mixture and subjected to 5 minutes at 55°C, 1 minutes of 94°C and 45 cycles of 1 second 94°C and 30 seconds at 57°C and read using FAM channel on a CFX96 (Bio-Rad Laboratories, Hercules, California). Each clinical sample was run with one technical replicate for each N1, N2, or RP assay, along with standards using synthetic RNA templates prepared in-house and quantified using ddPCR as described (21). Cq and SQ values were exported from Bio-Rad CFX Maestro 1.1 software (version 4.1.2433.1219) using the RFU threshold of 50 across all datasets.

**Sequence analysis**

Genomic sequences of SARS-COV-2 were downloaded from GISAID.ORG (acknowledgements: Supplementary Tables S2-S8). Criteria for inclusion were: sequences with designation as a Variant of Concern (VOC) or Variant of Interest (VOI) filtered for completeness, high coverage, collection on or before June 14, 2021 and submitted prior to July 1, 2021. The first 1000 sequence records for each VOC / VOI in the GISAID.ORG database were used for subsequent analysis. This sequence library was screened for perfect identity matches with the primer binding regions of the NC1, NC2, NC3 assays using the packages Biostrings (22) and Seqnir for R (23).

**Results**

To efficiently combine mRT-LAMP assays and differentiate between target and IAC amplification in a crude sample matrix requires two key features: a target specific probe technology (Fig. 1) and a strand displacement polymerase with low non-template amplification. We developed fluorescent universal displacement probes (UDP)s to allow multiplexed assays to be combined or parsed into fluorescence channels with a minimum number of probes. UDPs themselves are engineered sequences that use a universal adapter sequence on a loop primer for target-specific detection (Fig. 1A). In the configuration presented here, three independent SARS-CoV-2 targets are designed to report to a single
green (6-FAM) fluorescent probe, and the IAC is designed to report to a red (TEX615) fluorescence channel (Fig. 1B). We previously developed an in-house thermostable strand displacement polymerase (TFpol) with very low nonspecific amplification that is amenable to multiplexing. The TFpol design was inspired by the chimeric polymerase method of Morant (24) using the polI polymerase of *Thermus Thermophilus* as the backbone, an enzyme shown to be tolerant of many polymerase inhibitors (25, 26). UDPs and TFpol combine to allow for a flexible and robust mLAMP system, compatible with multiple target redundancy, IAC controls, and potential for reduced sample preparation.

Figure 1: Multiplexed RT-LAMP (mRT-LAMP) fluorescence detection by Universal Displacement Probes (UDP). A) UDP incorporation during LAMP amplification and activation by displacement of quenching strand. Primer and probe refer to loop (L), adapter (A), and quencher (Q), with complementary sequences denoted with the suffix “c” (e.g., “Lc” is the reverse complement “L”). B) Two-channel fluorescence detection of multiplexed redundant LAMP products (6-FAM) and shared-primer IAC (TEX615) by UDPs. Primer designations refer to forward (F), backward (B), and loop (L) using conventional LAMP terminology.

Analytical performance of SARS-CoV-2 mRT-LAMP

Functionality of the individual redundant targets in the mRT-LAMP was verified using synthetic RNA fragments corresponding to NC1, NC2, or NC3 mRT-LAMP assay footprints. All three target regions generated detectable amplification (Supp. Fig. S1A) with similar average reaction times with 200 copies of transcript RNA (NC1: 26.4 min, NC2: 26.3, NC3: 28.7 min; Supp. Fig. S1B).

The multiplexed assay was evaluated with synthetic target RNA containing all three target regions in the presence of 105 copies of a single-stranded DNA internal amplification control (Fig. 2A). The amount of IAC was chosen to allow detection of low-copy targets prior to detection of the IAC, in order to reduce resource competition between target and control amplifications. This timing differential is possible because of the reduced rate of amplification with a single loop primer in the IAC primer set, when compared to the target assays with a standard complement of LAMP primers. Input of 200 SARS-CoV-2 RNA copies (Fig. 2A, top) resulted in detection of green fluorescence in about 21 minutes, while the IAC was not detected. For zero SARS-CoV-2 input copies, there was no target amplification, and the IAC signal was detected by red fluorescence at about 27.5 minutes (Fig. 2A, bot). This behavior is ideal for a shared-primer IAC strategy, permitting detection of the target organism or, alternatively, validating the assay chemistry with the control reaction in the absence of target NAs. The analytical sensitivity was assessed with synthetic RNA target (Fig. 2B). All reactions containing target RNA were positive, and all NTC reactions detected IAC amplification and were negative for target (Fig. 2A). Some IAC amplifications were detected in low copy reactions containing target RNA (Fig. 2A), but their presence did not compromise target detection. The assay detected down to 5 copies per reaction (n=4), and all reactions had threshold times of 30 minutes or less for both the target and IAC (Fig. 2C).
Figure 2: Analytical performance of mRT-LAMP for SARS-CoV-2. A) Characteristic amplification of multiplexed SARS CoV-2 target and internal amplification control (IAC) with real-time fluorescence detection by universal displacement probes (UDP). Single representative run with 200 copies of synthetic RNA input or a no template control (NTC). B) Analytical sensitivity of multiplexed SARS-CoV-2 target and IAC. IAC amplifications (bottom) correspond to target amplifications (top). Target synthetic RNA input: 2,000 (n=3), 200 (n=3), 20 (n=3), 10 (n=3), or 5 copies per reaction (n=4); and NTC (n=3). C) Time to detect signals from SARS-CoV-2 and IAC for reactions from panel B.

Tolerance to transport media

To evaluate the tolerance of the assay to potential media contaminants, a selection of commercially available co buffered transport reagents were spiked into reactions with a 25% final concentration. For a 20µL total reaction volume, 5µL of 1x DMEM (11965-06, Gibco), 1x VTM (BD 220527, Copan), 1x PBS (SH30256.01, GE) or 0.9% sodium chloride (diluted from 5M stock 71386-1L, Sigma) was added into the mRT-LAMP reactions with final synthetic SARS-CoV-2 RNA of 0, 20, or 200 copies (Supp. Fig. S2). Successful SARS-COV-2 amplification was observed for all samples containing template under all buffer conditions.

Performance with extracted clinical specimens

The SARS-CoV-2 mRT-LAMP was evaluated against a collection of pre-extracted patient specimens. Of the 102 samples evaluated by RT-PCR, 93 were determined to contain human origin material by positive RNase P (RP) results; all samples that were negative for RP were also negative for SARS-CoV-2 and were considered indeterminate. Of the 93 specimens verified to contain human material, 60 were found to be negative for SARS-CoV-2, and 30 were found to be positive by both reference RT-RCR assays. The three remaining samples were positive for SARS-CoV-2 by one reference RT-PCR assay and negative by the second, resulting in an inconclusive classification. All samples that were indeterminate or inconclusive by RT-PCR were excluded from analysis. Clinical samples were run in duplicate mRT-LAMP reactions.

The mRT-LAMP was able to detect negatives with 100% specificity in both sets of replicates, with detection of the IAC but no target signal (Fig. 3). Conversely, sensitivity for the two replicates was 90% (27/30) and 87% (26/30), respectively. For samples found to have more than 30 copies / mRT-LAMP reaction by reference RT-PCR, sensitivity was improved to 100% (21/21) for both replicates. The OpenArray characterization of the verified samples found 57 of 90 validated samples contained one or more other respiratory infections; 8 of 9 SARS-CoV-2 positive samples with coinfections were correctly called as SARS-CoV-2 positive, 48 of 48 SARS-CoV-2 negative samples that were positive for other pathogens were correctly called as SARS-CoV-2 negative.

Figure 3: mRT-LAMP amplification of extracted nasal specimens. Samples confirmed positive or negative for SARS-CoV-2 and positive for the RNase P human marker by RT-PCR panel (N1, N2, RP) were amplified by duplicate mRT-LAMP reactions. Detected mRT-LAMP signals for SARS-CoV-2 (CoV) are shown in blue, and IAC signals are shown in orange; replicate pairs are connected by a line segment. Mean copy number was derived from qPCR results of N1, N1 PCR (see Supplemental Table S1).
Primer coverage analysis

Individual primer sets had variability in the frequency of perfect primer matches across the VOC/VOI sequence libraries (Table 2). Presence of mismatches does not necessarily preclude assay functionality and is therefore an underestimate of realized assay coverage. Combined primer coverage, where one or more primer sets had a perfect match for the target, was high across all variants, with a minimum 97.4% (for Eta).

Table 2: Coverage of Variant Sequences by individual and multiplexed targets

| SARS-COV-2 VOC/VOI | NC1 Primers | NC2 Primers | NC3 Primers | multiplex coverage (1+ primer set match) |
|--------------------|-------------|-------------|-------------|----------------------------------------|
| Alpha              | 71.8        | 90.0        | 79.9        | 100.0                                  |
| Beta               | 96.4        | 90.4        | 95.9        | 100.0                                  |
| Delta              | 96.7        | 96.5        | 0.0         | 99.8                                   |
| Epsilon            | 94.6        | 93.7        | 81.2        | 99.6                                   |
| Gamma              | 0.5         | 80.7        | 88.6        | 98.8                                   |
| Eta                | 93.9        | 0.4         | 83.7        | 97.4                                   |
| Iota               | 88.0        | 81.5        | 92.5        | 99.9                                   |

Discussion

This initial validation of a multiplexed reverse transcription LAMP assay is a further step towards more resilient point-of-care NAAT technologies with convenient implementation and development. The assay supports robust but basic functionality with competitive sensitivity, speed, and a low complexity fluorescence detection system. Because VTM has been shown to inhibit conventional PCR strategies (27), we designed our assay to use our in-house chimeric polymerase, TFpol, which has been proven to be effective with complex samples containing various transport media. TFpol supports multiplexed LAMP amplifications which have been infrequently demonstrated. These capabilities, taken together, enable features that are contemporary in high–throughput laboratory testing but more challenging in point-of-care diagnostics.

Multiplexed LAMP reactions with the ability to differentiate individual products by target specific probes enable two key aspects of robust NAAT testing: internal amplification controls and multiple target redundancy. IACs are widely accepted as a means of assuring the sample could detect a positive result if the target result is negative, by verifying that the reaction chemistry was viable and not inhibited by sample contaminants or otherwise compromised (28). In the context of LAMP amplification, internal controls can impair successful target detection; the resource demands of a successful LAMP
mean co-amplification of multiple products with varying inputs often lead to the competitive inhibition of slower assays or lower concentration of target (Fig. 2B, 2C). Presumably, this can be attributed to resource depletion of limiting reagents in the reaction mix. To address this resource competition, we devised a shared-primer internal control strategy where the performance of the IAC has been intentionally impaired by using a reduced primer set. The delayed time-to-detection of the IAC can then be further controlled by adjusting the concentration of control template, ensuring reduced competition with the target amplification.

The single-pot multiple target redundancy is a defining feature of this assay design even with the grouped reporter signal. Pathogen genetic variability is an important failure mode for nucleic acid amplification tests; a single nucleotide point mutation (SNP) can result in underperformance of a LAMP (29) or PCR reaction (30). As the COVID-19 pandemic progresses, the virus will continue to accumulate mutations and diversify, posing a challenge to NAATs used for diagnosis. An alignment of publicly available SARS-CoV-2 genomes at the time of writing reveals multiple genomes with known mutations in the primer footprints of the CDC PCR designs and a range of other published assays, suggesting that mutations are an existential problem (data not shown). The likelihood of these mutations rendering all three target amplifications ineffective simultaneously is lower than for a single assay. This principle is often incorporated in commercially available conventional laboratory based NAATs so this capability represents a convergence of state-of-the-art diagnostic methods and POC diagnostic capabilities. Our own analysis (Fig. 2) found that, since the design of the multiplex assay in early 2020 against the NCBI reference sequence, the emergence of the many variant lineages had resulted in some high frequency mismatches in primer binding regions of our targets. While many single nucleotide polymorphisms are likely to be tolerated by a LAMP reaction, some mutations, such as those located at or near the critical termini of primers, may interfere with diagnostic performance (31). Variants Delta, Gamma and Eta each had fixed, or almost fixed allele mutations in primer sets NC3, NC1, and NC2 respectively. When considered in combination as a multiplex assay, the primers still showed good overall coverage despite one of the three assays being potentially compromised; this is a clear demonstration of the value that multitarget redundancy holds for viral diagnostics. In the case of the Delta variant, which currently has rising incidence (32), a sequence mutation within the NC3 primer set was identified on the reverse bumper primer (B3, data not shown). This primer could be altered to be inclusive to the delta variant or redesigned without disruption to the diagnostic, ensuring no lapse in diagnostic coverage over time.

In order to fully realize a field ready POC assay additional development is planned. While preliminary testing suggests that the system is tolerant to inhibitors that might typically interfere with a direct-to-amplification workflow, in-amplification sample lysis, and testing with human sample matrix will be necessary. This is particularly important for understanding the role of RNAses on assay sensitivity. With the current ssDNA IAC design RNA integrity is not assessed by the control system, which is essential for a fully functional process control. Future iterations will address this by implementing an encapsidated RNA, such as MS2 coliphage for this purpose. Sampling, storage, and portable device solutions are already under development (13). These advancements will allow us to eschew infrastructure requirements that have acted as a bottleneck in current testing efforts, and when combined with the robust multiplex chemistry presented here, could act as a practical solution for decentralized testing.

**Author Contributions**

E.K. designed this unique version of LAMP assay and in-house polymerase. E.K., I.T.H., and Q.W. prepared the in-house polymerase and synthetic RNA targets. N.P. and E.K. performed mRT-LAMP experiments. N.P. and A.K.O. performed extraction of clinical specimens, RT-qPCR. E.K.
performed the analysis of mRT-LAMP and RT-qPCR. P.D.H. and L.M.A. designed the specimens panel used in this study and characterized these specimens using RT-qPCR and OpenArray. B.R.L. oversaw the study. All authors contributed to writing this manuscript.

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Conflicts of Interest

Patent applications have been filed on several components of this assay. E.C.K., N.P., Q.W., I.T.H., D.L. and B.R.L. are inventors on one or more provisional patent applications. E.C.K., N.P., Q.W., I.T.H., A.K.O, and B.R.L. have equity in a startup company that licenses this technology.

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