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Multiplexed and Extraction-Free Amplification for Simplified SARS-CoV-2 RT-PCR Tests

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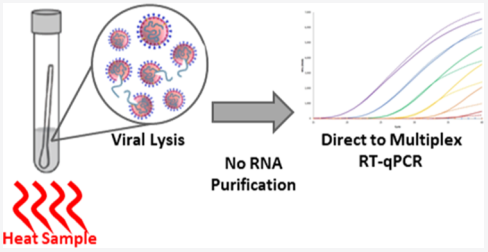
ABSTRACT: The rapid onset of the global COVID-19 pandemic has led to challenges for accurately diagnosing the disease, including supply shortages for sample collection, preservation, and purification. Currently, most diagnostic tests require RNA extraction and detection by RT-PCR; however, extraction is expensive and time-consuming and requires technical expertise. With these challenges in mind, we report extraction-free, multiplexed amplification of SARS-CoV-2 RNA from 246 clinical samples, resulting in 86% sensitivity and 100% specificity. The multiplex RT-PCR uses the CDC singleplex targets and has an LoD of 2 c/μL. We also report on amplification using a range of master mixes in different transport media. This work can help guide which combinations of reagents will enable accurate results when availability of supplies changes throughout the pandemic. Implementing these methods can reduce complexity and cost, minimize reagent usage, expedite time to results, and increase testing capacity.



In December 2019, a pneumonia of unknown cause was detected in Wuhan, China and reported to the WHO.[1] The novel coronavirus disease (COVID-19) spread rapidly on a global scale. At the time of this writing, there are over 98 million cases of COVID-19 and 2 million deaths reported to the WHO with numbers increasing daily.[2] In January 2020, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was confirmed as the infectious agent, and the full genome sequence was published shortly after,[3] enabling the development of critical diagnostic tools.

Currently, the majority of authorized diagnostics require RNA extraction from respiratory tract specimens and detection by RT-PCR.[4] Although effective, RNA purification kits are expensive and add complexity and time to the diagnostic workflow. Most concerning, months into the COVID-19 pandemic, testing remains dangerously inadequate in many countries in part due to shortages of test materials. Additionally, many diagnostics require running three singleplex reactions per sample,[5] limiting testing throughput. Streamlining the diagnostic workflow to reduce complexity, time, and reagent usage could increase testing consistency and capacity. Specifically, removing RNA extraction and identifying alternative reagents that demonstrate equivalent performance to current COVID-19 diagnostics could increase reagent options and alleviate known bottlenecks. Efforts are underway by multiple groups to achieve these goals.

Direct amplification of SARS-CoV-2 from patient swabs in simple buffers such as phosphate-buffered saline[6] or TE (Tris, ethylenediaminetetraacetate)[7] or from saliva samples[8] has been demonstrated. Although promising, these workflows do not account for the current clinical practices of collecting swabs in viral transport media (VTM) prior to testing. Beltrán-Pavez et al. demonstrated direct amplification from VTM but only tested a single source, which resulted in significant amplification inhibition.[9] Sample collection sites often use multiple types of VTM depending on current availability; therefore, improvements to diagnostic test workflow should explore a variety of transport media, including options that can easily be made in-house. The CDC has an authorized standard operating procedure (SOP) for preparing VTM (SOP#: DSR-052-02. 2020) from readily available reagents when commercial sources are unavailable.[10]

Smyrlaki et al. reported promising results for extraction-free SARS-CoV-2 detection from nasopharyngeal (NP) swabs,[6] but their method relies on multiple singleplex tests which reduce potential throughput. Also, their reported data require only a single positive SARS-CoV-2 target to identify a positive sample. This does not align with the majority of CDC or WHO recommendations, which require at least two SARS-CoV-2 targets to determine positivity.[5,11]

Given the current landscape of SARS-CoV-2 diagnostics and the need to increase testing capacity, we have explored directly amplifying SARS-CoV-2 target from multiple VTMs in combination with testing five RT-PCR master mixes (MMs)

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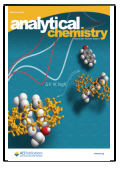

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from different manufacturers.[12−14] Four of the five MMs are authorized for use with the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel,[5] and one has shown encouraging results for direct RT-PCR amplification from clinical samples.[15] Additionally, we demonstrated a multiplex RT-PCR to compare the three singleplex reactions required currently[16] (referred to as the "CDC singleplex test" from here on). Over 60 molecular COVID-19 diagnostics have received FDA emergency use authorization since the CDC singleplex test was implemented.[17] Multiplexing of the CDC targets has been demonstrated;[18] however, multiple of these tests require specialized equipment[19] and specific MMs,[20] limiting reagent options.

We demonstrate that direct amplification from multiple VTMs paired with a multiplex RT-PCR using the CDC singleplex targets is equivalent to approved, more complex diagnostic protocols. This multiplex has a limit of detection (LoD) of 2 copies/μL input (c/μL). Additionally, we evaluated this method using 246 clinical samples, resulting in 86% sensitivity and 100% specificity. This approach improves the current diagnostic workflow by reducing complexity, shortening time to results, increasing throughput, and alleviating reagent shortages. The improvements and findings described here may be suitable for other pathogen-detection applications.

EXPERIMENTAL SECTION

See the Supporting Information for more detailed materials and methods.

Clinical Samples. A total of 354 de-identified samples were received from four sources. Most samples arrived as NP swabs in VTM; our research team had no control over the various VTMs used or the sample storage conditions prior to receiving them. RNA was purified from each sample prior to testing to determine RNA loads.

The Washington State Department of Health (DOH) Public Health Laboratories (Shoreline, WA, USA) provided 120 NP swab samples in VTM, which included 60 SARS-CoV-2 positives and 60 negatives. Matched purified RNA was provided for 30 of the 60 positive samples. Thirty additional negative samples were provided as purified RNA without the matched swab in VTM.

We also received 76 NP swab samples in VTM from BioIVT (Westbury, NY, USA) and 50 from Medix (Lombard, IL, USA). These samples included 45 SARS-CoV-2 positives (26 from BioIVT, 19 from Medix) and 81 negatives (50 from BioIVT, 31 from Medix). For all samples, SARS-CoV-2 viral loads were determined using the N1 target, and human RNA load was determined using the RP target. All samples were discarded and de-identified and therefore did not require IRB approval.

We also received 78 clinical samples from Discovery Life Sciences (Los Osos, CA, USA), which were negative for SARS-CoV-2, but 17 were positive for a variety of other respiratory infections.

RNA Purification from Clinical Samples. For RNA extraction, either 70 or 140 μL of sample was purified using the QIAamp Viral Mini Kit according to the manufacturer's protocol.[21] The purified RNA was eluted in 140 μL of provided elution buffer according to CDC recommendations.[16]

In Silico Analysis. All CDC primers and probes sequences were analyzed in Geneious Prime version 2020.0.3 and screened for unfavorable folding and interactions using AutoDimer Version 1.0[22] with the following parameters: Minimum SCORE Requirement: 3; Na+ 0.085 M; temp for dG calc 37 °C; total strand conc 1.0 μM.

Singleplex and Multiplex RT-PCR for SARS-CoV-2. All RT-PCRs were run using the BioRad CFX96 Real-Time PCR Detection System (Hercules, CA, USA) with fluorescent data collected during the annealing step. All primers and probes sequences are available in Table S1, and the protocols for each MM are outlined in Table 1. For the multiplex test, we tested various primer concentrations and additional probe-fluorophore combinations to optimize the assay.

Table 1. Overview of RT-PCR Protocols for Each MM Tested[a]

master mix	initial step	reverse transcriptase	initial denaturation	thermal cycling (45×)
TaqPath, ThermoFisher	25 °C, 2 min	50 °C, 15 min	95 °C, 2 min	95 °C 3 s, 55 °C 30 s
GoTaq, Promega		45 °C, 15 min	95 °C, 2 min	95 °C 15 s, 55 °C 60 s
UltraPlex, QuantaBio		50 °C, 10 min	95 °C, 3 min	95 °C 3 s, 55 °C 30 s
qScript, QuantaBio		50 °C, 10 min	95 °C, 1 min	95 °C 3 s, 55 °C 30 s
LunaScript, NEB		50 °C, 10 min	95 °C, 1 min	95 °C 10 s, 55 °C 30 s

[a]All reactions included 45 cycles of denaturation and annealing based on the recommendation from the CDC for the SARS-CoV-2 N1 and N2 targets.[16] The protocols were the same for the singleplex and multiplex reactions.

Impact of VTM on RT-PCR. The impact of three VTMs (S2, M4RT, and GG-VTM) on five different RT-PCR MMs was evaluated by spiking control SARS-CoV-2 and human DNA plasmids (from IDT) into stock and diluted VTM. The final concentration of SARS-CoV-2 target in each sample ranged from 0.8 to 820 c/μL, and the final concentration of human target was held constant at 2000 c/μL.

Impact of Thermal Lysis on SARS-CoV-2 Detection Direct from Patient Samples. The impact of thermal lysis on the detection of SARS-CoV-2 from 60 patient samples was determined by comparing four different conditions for each sample. A total of 246 clinical samples were tested for SARS-CoV-2 detection by heating to 98 °C for 5 min, followed by amplification in the multiplex RT-PCR. RNA was also purified from each sample to determine the COVID-19 status.

RESULTS AND DISCUSSION

In Silico Analysis. In silico analysis did not predict unfavorable interactions between any primers and probes. These results suggest that the singleplex reactions could be combined into a multiplex reaction.

Developing a Multiplex RT-PCR for SARS-CoV-2. Purified SARS-CoV-2 target was used to evaluate the performance of a multiplex RT-PCR, consisting of N1, N2, and RP targets from the CDC singleplex test. Validation was performed with a 1:1 ratio of SARS-CoV-2 to human target and with low copies of SARS-CoV-2 target with increasing copies of human target, Figure S1.

For this multiplex reaction, we observed unexpected results for the N2 target when increasing the amount of human target in the reaction. When the SARS-CoV-2 concentration was held constant, the *Ct* appeared to decrease as the human target

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Table 2. LoD Analysis[a]

target	10 c/μL	8 c/μL	6 c/μL	4 c/μL	2 c/μL	1 c/μL	0.4 c/μL
				Multiplex Assay			
N1	20/20 (33.8)	20/20 (34.4)	20/20 (34.4)	19/20 (35.3)	19/20 (36.8)	11/20 (37.6)	11/20 (38.0)
N2	20/20 (32.7)	20/20 (33.2)	20/20 (33.3)	20/20 (34.2)	20/20 (34.3)	14/20 (34.7)	15/20 (35.7)
				CDC Singleplex Assay			
N1	20/20 (30.1)		20/20 (31.0)	20/20 (32.3)		20/20 (34.5)	7/20 (39.2)
N2	20/20 (32.4)		20/20 (32.4)	20/20 (34.5)		20/20 (35.2)	0/20 (NA)

[a]LoD of our multiplex test and the CDC singleplex test performed side-by-side, both assays included 10^4 c/μL human target. The singleplex LoD results reported here match those published by the CDC.[16] Each assay was run with 20 replicates and average *Ct* values are reported based on the positive wells. All reactions were run using the qScript MM from QuantaBio.

concentration increases. We believe this to be signal carryover from the human RP target in the Cy5 channel when using the N2-TexasRED probe, Figure S1C. Due to these initial results, several improvements were made to the multiplex reaction.

N2 performance was originally the weaker of the two viral targets, showing much lower signal-to-noise and a more stochastic trend at higher input than the N1 target. To improve the signal-to-noise of the N2 target, a brighter fluorophore, AlexaFluor594, was used, and the primers and probe concentrations were increased to facilitate performance at lower input amounts. The best performance was observed with final concentrations of 2000 nM of each N2-primer and 500 nM of the N2-AF594 probe. These improvements did not drastically improve the LoD of the reaction, but the N2 signal was more reliable, producing a more significant increase over background when positive. Additionally, at lower SARS-CoV-2 concentrations, the N2 signal no longer appears influenced by carryover from the Cy5 channel. The final concentrations in the reaction for the primers and probes were 500 nm and 250 nM (N1), 2000 nM and 500 nM (N2), and 500 nM and 250 nM (RP).

Using the optimized reaction, we tested the specificity of the multiplex RT-PCR in TaqPath MM using RNA purified from 78 SARS-CoV-2-negative patient samples, including 17 that were positive for a variety of other respiratory infections. None of these samples showed false-positive results when tested with the multiplex reaction, Table S2.

The Washington State DOH provided purified RNA from 90 patient samples to compare the performance of the multiplex assay to the CDC singleplex test. We observed 86% concordance with SARS-CoV-2 positive samples and 98% concordance with negative samples, Table S3. We observed that the N2 signal was undetectable in the multiplex reaction more frequently than in the CDC singleplex test with these samples. In general, the N2 target resulted in higher *Ct* values than the N1 target for the same sample in singleplex and multiplex reactions. On average, the N2 *Ct* value was 0.74 *Cts* higher than N1, and 27 of 30 positive samples had higher N2 *Cts* than the corresponding N1 target. Based on this difference in N1 and N2 *Ct* values, it is likely that the N2 *Ct* fell below the detection threshold for the multiplex assay, resulting in false negatives.

All the multiplex false-negative results detected the N1 but not the N2 target. We reported a sample positive only if both N1 and N2 were detected to align with the recommendations from the CDC singleplex test.[5] Currently, two commercial assays[24,25] and the CDC COVID/FluAB multiplex test require only a single SARS-CoV-2 target for determining a positive sample. If our data used these guidelines, the positive concordance would slightly improve, but it would also add additional false-positive samples, decreasing the negative concordance with the CDC singleplex. Additionally, for all the false-negative samples, the N1 *Ct* value was very high (above 36) and N2 was not detected, which is likely attributed to RNA degradation after freeze/thaw cycle(s). This is a well-reported observation for RNA in general[26−28] and recently for SARS-CoV-2 RNA.[6]

The purified RNA from DOH to validate the multiplex reaction experienced at least one freeze/thaw cycle prior to our tests. To assess potential RNA degradation, we used the CDC singleplex test to compare the *Ct* values provided by the DOH to the *Ct* values after samples were received and experienced at least one freeze/thaw. On average, the N1 values were 1.7 *Cts* higher after freeze/thaw compared to the fresh DOH reaction, and 25 of 30 samples resulted in higher *Ct* values post freeze/thaw. This same trend was observed for the RP target. On average, the RP *Cts* were 3.0 higher after freeze/thaw and 29 of 30 samples resulted in higher *Ct* values post freeze/thaw. The impact on both SARS-CoV-2 and human RNA suggests that the degradation was not specific to the viral RNA.

LoD Determination of the SARS-CoV-2 Multiplex Assay. Using the optimized multiplex assay, 20 replicates of SARS-CoV-2 target at 0.4, 1, 2, 4, 6, 8, and 10 c/μL with 10^4 c/μL human control target were tested to assess the assay LoD, Table 2. We also performed the CDC singleplex reactions with 20 replicates of SARS-CoV-2 target at 0.4, 1, 4, 6, and 10 c/μL with 10^4 c/μL human control target as a direct comparison. All reactions were run using the qScript MM from QuantaBio.

For the multiplex reaction, the LoD was 2 c/μL; at this input, N1 was detected 19/20 and N2 20/20. When 1 c/μL of sample input was used, N1 was detected 11/20 and N2 14/20. With 0.4 c/μL of sample input, N1 was detected 11/20 and N2 15/20. For the singleplex reactions, the LoD was 1 c/μL, which matches LoD reported by the CDC.[16] Samples were considered positive if *Ct* values were below 40, as recommended by the MIQE guidelines.[23]

Direct Amplification of RNA from VTM. Currently, there are reagent shortages throughout the diagnostic workflow, including VTMs and RT-PCR MMs. Due to these constraints, clinical collection sites often use a variety of VTMs based on availability. With this in mind, three common VTMs were tested in five commercially available RT-PCR MMs to determine the impact on SARS-CoV-2 detection.

Purified SARS-CoV-2 and human control plasmids (from IDT) were spiked into VTM or VTM diluted in 10 mM Tris at pH 8. The fold-change in *Ct* values was calculated to determine the impact on amplification (see Supporting Information). If a condition did not have a *Ct* value, it was set to 45, the maximum number of cycles, to determine a Δ*Ct*. We tested all VTM/MM combinations with a range of SARS-

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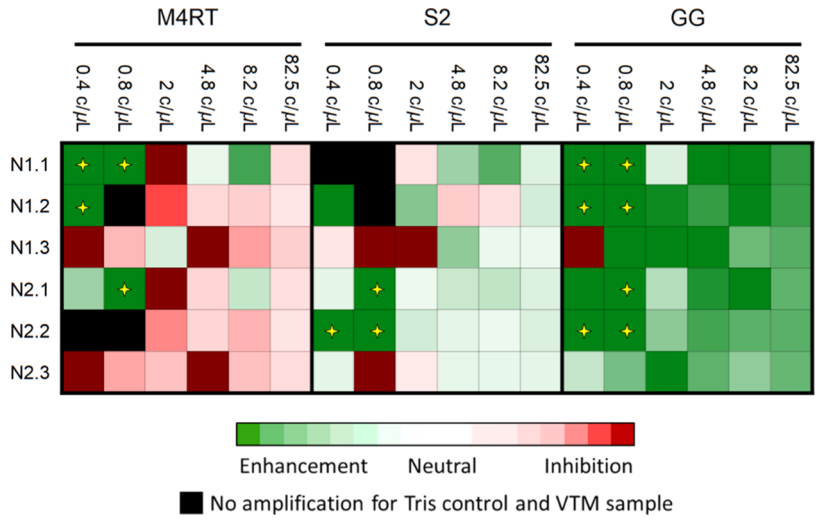

Figure 1. Impact of VTM on the multiplex assay using the qScript MM. The data presented are fold-change in *Ct* values between SARS-CoV-2 target spiked into VTM and control reactions of target spiked in 10 mM Tris buffer, pH 8. Three replicates are shown for each condition. The black boxes indicate samples where the amplification signal was not detected for both the Tris control and VTM sample. Conditions marked with a "✦" represent signal in the VTM sample, but no signal in the Tris control sample, suggesting amplification enhancement.

CoV-2 and human target concentrations using the CDC singleplex test. The UltraPlex and qScript MMs were the most tolerant and showed amplification enhancement in two of the three VTMs. The M4RT VTM resulted in the most amplification inhibition, significantly impacting three of the five MMs, Figures S2−S5.

Amplification in the qScript MM accurately detected both N1 and N2 targets at very low input of SARS-CoV-2, Figure 1. Unsurprisingly, amplification was not observed for all replicates of the Tris control or VTM samples with inputs at or below 0.8 c/μL. In a few cases, the VTM samples resulted in amplification when the Tris controls did not, suggesting amplification enhancement in the presence of some VTM. Amplification enhancement was confirmed by performing an LoD experiment using N = 20 replicates with targets spiked into VTM, Table S4. The same experiment was performed with the TaqPath MM, which showed significant amplification inhibition of across all VTMs tested (data not shown).

Most COVID-19 clinical samples are stored in VTM prior to testing, but due to reagent shortages, clinical collection sites often rely on multiple VTM sources. For the clinical samples used in this study, we had no control over which VTM was used or the storage conditions prior to receiving samples. With this constraint in mind, the ideal COVID-19 tests would be tolerant of multiple VTMs to reduce the need for sample clean-up prior to testing. Based on the results presented here, the qScript and UltraPlex MMs perform well in combination with all tested VTMs and low SARS-CoV-2 input. These MMs were formulated to be ultratolerant to inhibitors and enable co-amplification of low copy targets in the presence of higher copy reference genes.[29] Data from mock samples of target spiked into VTM suggest that purification is not necessary for COVID-19 detection.

From the clinical samples we received, the SARS-CoV-2 positives had viral loads that ranged between 1.9×10^{-1} and 6.5×10^{9} c/μL with a median value of 1.3×10^{3} c/μL (N = 96, Supporting Information). These clinical viral load data paired with the low detection limit of the multiplex RT-PCR suggest that SARS-CoV-2 can be amplified directly from clinical samples without purification.

Therefore, we tested extraction-free amplification from clinical samples using the multiplex assay. This simplified workflow reduced the time-to-result for each sample by over an hour due to the removal of the RNA purification step. Using 246 clinical samples (96 positive and 150 negative), we tested 98 °C heating for 5 min, followed by direct multiplex amplification. The COVID-19 status was determined from multiplex amplification of in-house-purified RNA from each clinical sample. Overall, the simplified workflow resulted in 86% sensitivity and 100% specificity for all clinical samples (NP swabs in VTM), Table 3.

Table 3. Extraction-Free Detection of SARS-CoV-2 from Clinical Samples Using the Multiplex RT-PCR[a]

	total	$\geq 10^3$ c/μL	10^2 c/μL	10^1 c/μL	$\leq 10^0$ c/μL
positive	82/96 (86%)	47/47 (100%)	19/22 (86%)	8/10 (80%)	8/17 (47%)
negative	150/150 (100%)				

[a]The sensitivity and specificity of the simplified workflow are 86 and 100%, respectively. The positives are also grouped by viral load. The COVID-19 status of each sample as "positive" or "negative" was determined using RNA purified in-house for each sample. For each sample, three replicates were averaged to determine the outcome below. Samples were considered positive if *Ct* values were below 40, as recommended by the MIQE guidelines.[23]

As detailed above in Table 3, lower SARS-CoV-2 concentrations resulted in less accurate detection. When using all 246 clinical samples, the extraction-free, multiplex test had a sensitivity and specificity of 86 and 100%, respectively. When the 17 samples with viral loads of 10^0 c/μL are excluded, the specificity remains at 100%, while the sensitivity increases to 95%. As previously noted, the extraction-free method shows higher *Ct* values for a sample compared to purification prior to amplification. This efficiency hit significantly impacts the samples with very low viral loads causing the extraction-free assay to only detect 8/17 at the low end. This work used discarded and de-identified clinical sample remnants that had gone through at least one (but likely

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multiple) freeze/thaw cycles prior to our testing. RNA degradation due to freeze/thaw cycles has been well-documented[6,26,27] and shows a greater impact on samples with lower viral loads because they are near or at the detection limit for an assay. The CDC singleplex test was not explicitly validated with frozen/thawed samples, even though the many clinical samples are tested post freeze/thaw. Smyrlaki et al. demonstrated extraction-free amplification with the CDC singleplex assays and reported high sensitivity and specificity (96 and 99.8%, respectively) but did not report the sample viral loads or whether the samples were fresh or frozen.[6] Future work should explore the impact of RNA degradation on our extraction-free workflow and the sensitivity of this assay by comparing detection from fresh and frozen samples directly. This work could be coupled with exploring the addition of RNase inhibitors to VTM prior to sample collection to help reduce potential RNA degradation from the matrix itself. This was not explored for this work because it would require an additional reagent that may be challenging to source.

Recently, there have been reports of up to 33% false negative results with FDA authorized systems such as the Abbott ID NOW.[30] One of the most attractive features of this test is the simplified workflow: NP samples added directly to the test. Our data also demonstrate a simplified workflow by directly amplifying samples from NP swabs, and it enables more flexibility in reagent selection and shows significantly increased specificity.

Impact of Thermal Lysis on SARS-CoV-2 Detection Direct from Patient Samples. Finally, we tested a subset of 60 clinical samples to compare the impact of a thermal lysis step at either 65 °C for 30 min or 98 °C for 5 min to a no lysis condition. Clinical samples were determined to be positive if SARS-CoV-2 RNA was detected after RNA purification in-house.

Thermal lysis at 98 °C for 5 min prior to amplification improved the detection of SARS-CoV-2-positive samples (81%) compared to lysis at 65 °C for 30 min (56%) or no lysis (52%), Table 4. Only one false positive was observed across all conditions.

Table 4. Comparison of Lysis Conditions with Clinical Samples for SARS-CoV-2 Detection[a]

	no lysis	65 °C 30 min	98 °C 5 min
positive	14/27 (52%)	15/27 (56%)	22/27 (81%)
negative	32/33 (97%)	33/33 (100%)	33/33 (100%)

[a]"Positive" or "negative" status is determined by purifying RNA from each sample in-house and running it in the same multiplex RT-PCR as the different lysis conditions. If the purified RNA has detectable signal for N1 and N2, then it is positive. For all samples, each condition was run five times, and results were averaged to determine the outcome below. Samples were considered positive if *Ct* values were below 40, as recommended by the MIQE guidelines.[23]

In general, we observed an increase in *Ct* values for the viral targets across each condition when compared to the *Ct* values from purified RNA for the same sample. The increase in *Ct* was related to the detection accuracy reported in Table 4. For the no lysis and 65 °C heating conditions, the average increases in *Ct* values for the N1 target compared to purified RNA were 6.7 and 6.6, respectively. The 98 °C heating condition showed better results for the N1 target with a 3.4 *Ct* increase compared to the purified RNA, but this still reflects a measurable impact compared to purified RNA. The increases in *Ct* values for the target compared to purified RNA were relatively similar to those observed for the N1 target (*Ct* increases for N2, no lysis: 6.04, 65 °C: 5.93, and 98 °C: 2.85). These results were not observed for the RP reference target, which resulted in a *Ct* decrease of −1.3 across all 60 samples (both positives and negative) and no observed difference across the conditions (no lysis: −1.23, 65 °C: −1.22, and 98 °C: −1.43). This effect has been observed by other groups for SARS-CoV-2 RNA detection[6] and may be a result of inefficient viral lysis or RNA release from the virion.

CONCLUSIONS

Overall, this work suggests that eliminating RNA purification and simplifying the RT-PCR amplification to a multiplex test can significantly reduce complexity, time, and costs for detecting COVID-19 with the appropriate selection of MM. On-going work will explore pathways to further streamline this workflow, expand testing to larger numbers of clinical samples to further validate the approach, and explore the impact of VMT on RNA integrity.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.0c03918.

Primer and probe sequences for each assay, comparison of the CDC singleplex test and multiplex reaction, testing the specificity of the SARS-CoV-2 multiplex RT-PCR, multiplex RT-PCR concordance with the CDC singleplex, impact of stock VTM on the CDC singleplex test, ΔCt values used to determine the fold-change, impact of VTM on the CDC singleplex test, and side-by-side LoD comparison(PDF)

Details on *Ct* values and viral loads for all clinical samples tested (XLSX)

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S.A.B. and R.G. contributed equally. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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