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Extraction-Free Rapid Cycle Quantitative RT-PCR and Extreme RT-PCR for SARS-CoV-2 Virus Detection

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Since the start of the coronavirus disease 2019 (COVID-19) pandemic, molecular diagnostic testing for detection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has faced substantial supply chain shortages and noteworthy delays in result reporting after sample collection. Supply chain shortages have been most evident in reagents for RNA extraction and rapid diagnostic testing. This study explored the kinetic limitations of extraction-free rapid cycle quantitative real-time RT-PCR for SARS-CoV-2 virus detection using the commercially available capillary-based LightCycler. After optimizing for time and reaction conditions, a protocol for sensitive and specific quantitative RT-PCR of SARS-CoV-2 RNA from nasopharyngeal swabs in <20 minutes was developed, with minimal hands-on time requirements. This protocol improves detection speed while maintaining the sensitivity and specificity of hydrolysis probe-based detection. Percentage agreement between the developed assay and previously tested positive patient samples was 97.6% (n = 40/41), and negative patient samples was 100% (40/40). The study further demonstrates that using purified RNA, SARS-CoV-2 testing using extreme RT-PCR, and product verification by melting can be completed in <3 minutes. Overall, these studies provide a framework for increasing the speed of SARS-CoV-2 and other infectious disease testing. *(J Mol Diagn 2021, 23: 1671—1679; https://doi.org/10.1016/j.jmoldx.2021.08.004)*

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the virus responsible for coronavirus disease 2019 (COVID-19). Since it was first reported in Wuhan, China,[1—3] the virus has spread globally, resulting in large-scale disruptions to travel and activities, placed extensive pressure on healthcare systems and employees, and caused significant increases in mortality and morbidity. Several methodologies, such as traditional RT-PCR methods,[4] reverse transcription (RT) loop-mediated isothermal amplification,[5,6] and CRISPR-Cas13— and CRISPR-Cas12—based assays,[7—10] as well as serologic testing, have been implemented for the diagnosis of COVID-19. However, RT-PCR has remained the gold standard for molecular diagnosis of COVID-19.

The current COVID-19 pandemic has shed light on several shortcomings in molecular diagnostic testing. Since the beginning of the pandemic, there have been significant supply chain shortages, from personal protective equipment to molecular diagnostic reagents. Several groups have addressed the shortages of RNA isolation kits by testing different strategies for extraction-free SARS-CoV-2 testing. To address supply chain issues revolving around RNA isolation/purification reagents, several groups have demonstrated the feasibility of extraction-free SARS-CoV-2

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testing.[11–14] These groups have shown largely concordant results between extraction-free RT-PCR testing compared with RT-PCR using extracted RNA.[11–14]

In addition to significant supply chain bottlenecks, time to results for testing has led to significant delays in diagnosis, necessary isolation/quarantine, as well as treatment. Initial demand during the first wave of COVID-19 in the United States resulted in waits of over 1 week, and during the latest winter wave of infections, time to results was 2 to 4 days for priority patients at large national reference laboratories. Additionally, hospitals have faced significant shortages of rapid (<1 hour) nucleic acid amplification tests to help triage patients. Although RT-PCR tests have been shown to have a very high specificity and sensitivity for SARS-CoV-2, clinical applications of this methodology are traditionally time-consuming and reagent-intensive. RT loop-mediated isothermal amplification has been leveraged in COVID-19 diagnostic platforms, including the Abbott ID NOW (Abbott Laboratories, Abbott Park, IL). However, several reports have suggested that this test has a high false-negative rate in patients with low virus levels.[15–17]

To address the issues with time to results of assays for SARS-CoV-2, previously developed methodologies for extremely rapid RT,[18] as well as PCR, were utilized.[19] Although studies using extreme PCR have focused on intercalating dye-based detection, this study shows that faster thermocycling is possible with hydrolysis probe-based detection by using increased primer concentrations. Because a significant bottleneck in the supply chain is related to RNA isolation reagents, previously published research regarding extraction-free SARS-CoV-2 testing was utilized,[11–14] which, when paired with rapid cycle quantitative RT-PCR (RT-qPCR) resulted in an assay that can be completed in 20 minutes with little hands-on time. Overall, this study explored the kinetic restraints and optimization of extraction-free RT-PCR SARS-CoV-2 testing and lays the groundwork for future studies to improve the speed of RT-PCR assays.

Materials and Methods

PCR Primers

N1 primers and probe were used for LightCycler-based assays (Roche, Basel, Switzerland), the SARS-CoV-2 Centers for Disease Control and Prevention (CDC) (Table 1). Primers for optimal extreme PCR require higher annealing temperatures than what is traditionally used in PCR. Primers for extreme PCR were designed using the National Center for Biotechnology Information Primer-BLAST suite with optimal annealing temperatures set to 67°C with an oligonucleotide concentration of 10,000 nmol/L and an optimal product melting temperature of 80°C. Primers were then blasted against all virus, bacteria, and human Reference Sequences using National Center for Biotechnology Information Primer-BLAST. Primer sequences for extreme PCR can be found in Table 1.

Table 1 Primers Used

Name	Sequence
CDC_N1_F	5′-GACCCCAAAATCAGCGAAAT-3′
CDC_N1_R	5′-TCTGGTTACTGCCAGTTGAATCTG-3′
CDC_N1_probe	5′-FAM-ACCCCGCATTACGTTTGGTGGACC-BHQ1-3′
Extreme_N2_F	5′-CATTGGCATGGAAGTCACACCT-3′
Extreme_N2_R	5′-CCAATTTGATGGCACCTGTGTA-3′

SARS-CoV-2 Standards

SARS-CoV-2 genomic RNA, isolate USA-WA1/2020 (BEI # NR-52285), SARS-CoV-2 heat-inactivated virus, isolate USA-WA1/2020 (BEI # NR-52286), and SARS-CoV-2 quantitative synthetic RNA (BEI # NR-52358) were obtained from BEI Resources (Manassas, VA).

PCR Master Mixes

For extreme RT-PCR, reactions were performed in 5-μL volumes containing 50 mmol/L Tris (pH 8.3), 3 mmol/L $MgCl_2$, 200 μmol/L of each deoxynucleotide triphosphate (deoxyadenosine triphosphate, deoxycytidine triphosphate, deoxyguanosine triphosphate, and deoxythymidine triphosphate), 500 μg/mL nonacetylated bovine serum albumin (Sigma-Aldrich, St. Louis, MO), 2% (v/v) glycerol (Sigma-Aldrich), 10.0 μmol/L of each primer, 1 U/μL TaqIT DNA polymerase (Enzymatics, Beverly, MA), 2× LunaScript RT Enzyme Mix (New England Biolabs, Ipswich, MA), and 2.5 μmol/L Syto9 (Life Technologies, Carlsbad, CA). Due to the high concentrations of polymerase and primers, reactions were prepared on ice to avoid nonspecific amplification and primer−dimer formation. For rapid PCR conducted on the capillary-based LightCycler 1.5 instrument (Roche), Luna Probe One-Step RT-qPCR 4× Mix with UDG (New England Biolabs) was used. Reactions were performed in 10-μL volumes containing 1× Master Mix, 5 μmol/L of each forward and reverse primer, and 250 nmol/L of probe.

Melting Curve Generation and Analysis

Melting curves were generated using an HR-1 (Idaho Technologies, Salt Lake City, UT). For experiments comparing relative product amounts, LED voltages were kept constant. High-resolution melting data were analyzed with a custom program written in LabVIEW 2018 software (NI, Austin, TX) and viewed as derivative melting curves.[20]

PCR Thermocycling Protocols

For the LightCycler-based assay, programed temperatures and times were the following, RT at 55°C for indicated times, followed by 45 cycles of 95°C for 0 seconds, and a combined annealing/extension at 63°C for indicated times. Programed ramp rates between PCR steps were 20°C/second for all experiments. For extreme PCR, a hot bath

(100°C) and a cool water bath (60°C) was used to change sample temperatures, similar to a previously described method.[19] The water baths were heated on electric hotplates with temperature monitoring using an Omega OMB-DAQ-56 USB data acquisition module and Type-T thermocouples (Omega 5SRTC-TT-T-40-36; Omega Engineering, Norwalk, CT). A stepper motor (Stepperonline, model #23HS41-1804S; OMC, Nanjing City, China), driven by a digital stepper drive (Stepperonline, DM542T; OMC) with pulse and direction signaling provided by an Arduino Uno R3 (SparkFun Electronics, Niwot, CO) rotated samples in a custom sample holder between each water bath in <0.2 seconds. The stepper motor was controlled using a custom LabVIEW 2018 program similar to software previously described.[19] A thermocouple (Omega type T precision fine wire thermocouple, 0.003-inch diameter with Teflon insulation; Omega Engineering) centered in a dedicated control tube with 5 μL of mock PCR mix overlaid with 1 μL of mineral oil was used to measure temperature and trigger stepper motion. Stepper motor motion was triggered at 84°C for denaturation and 67°C for annealing/extension to obtain desired temperature cycling profiles for 40 cycles. RT was performed for 30 seconds in the cool bath at 60°C prior to thermocycling. All reactions were conducted in standard Roche LightCycler capillaries.

LightCycler RT Optimization

The 10-μL reactions were prepared on ice. Indicated RT times were set on the LightCycler for 55°C followed by a 2-minute 95°C incubation for reverse transcriptase inactivation. Samples then proceeded directly into thermocycling on the LightCycler. Samples were then thermocycled as above with a 30-second annealing/extension step. For the 0-second RT time point, the RT step on the LightCycler was removed, and samples were immediately ramped to 95°C at 20°C/second.

LightCycler PCR Cycle Time Optimization

The 10-μL reactions were prepared on ice. RT was conducted for 10 minutes on the LightCycler at 55°C followed by a 2-minute incubation at 95°C. Samples proceeded directly into thermocycling with indicated annealing/extension times. For the 0-second time point, the hold time for annealing/extension at 63°C was set to 0 seconds.

Patient Specimens

Nasopharyngeal (NP) swabs were collected in 1 mL of universal transport medium (Becton Dickinson, Franklin Lakes, NJ). For spiked samples, indicated amounts of SARS-CoV-2 inactivated virus was added to normal saline and subsequently processed. Positive patient samples were kindly provided by the University of Washington clinical virology laboratory. Briefly, samples were provided on dry ice in universal transport medium or viral transport medium. All samples had previously tested positive using either the University of Washington SARS-CoV-2 real-time RT-PCR assay or Panther Aptima transcription-mediated amplification (TMA; Hologic, Marlborough, MA) for SARS-CoV-2. Quantification cycle (Cq) values for the University of Washington SARS-CoV-2 real-time RT-PCR assay and relative luminescence unit values for the Panther Aptima TMA were also provided. Briefly, samples were mixed in a 2:1 ratio with sample and 20% Triton-X100, and then vigorously vortexed for three pulses of 5 seconds each. Following vortexing, samples were centrifuged at 20,000 *g* for 15 seconds to pellet cell debris. A total of 3 μL of supernatant was then used for each reaction.

Statistical Analysis

GraphPad Prism software version 8.0 (GraphPad Software, San Diego, CA) was used for all graphs and statistical analyses. Error bars in all figures represent SEM, with N marked in each figure legend. For pure RNA samples, the average of three technical replicates is shown.

Results

LightCycler Reverse Transcription Optimization

Since RT can be performed faster than the manufacturer's recommend time,[18] the shortest amount of time required for efficient RT was explored. Although extreme PCR and extreme RT typically use primer concentrations in the range of 5 to 20 μmol/L,[18,19] 5 μmol/L primer concentrations were used because capillary-based LightCycler PCR cycle times (20 to 40 seconds) are much slower than those used in extreme PCR (<1.2 seconds). The longer cycle times on the LightCycler would not require increased polymerase concentrations that might necessitate further increases in primer concentration. Luna Warm Start Reverse Transcriptase (New England Biolabs) was utilized because of its aptamer-based warm start inhibition, the reversibility of its warm start formulation, as well as its increased thermostability. The CDC SARS-CoV-2 assay N1 primer and probe set was used as a proof of principle to further generalize the results.

Previous studies examining rapid RT primarily utilized isolated RNA.[18] To optimize this assay for extraction-free testing, the time requirements for RT were examined with pure RNA, and SARS-CoV-2 genomic RNA was spiked into normal saline, as well as 10 samples, for which the patients had tested positive (prepared as in *Materials and Methods*). After setting up reactions on ice, the reaction tubes were incubated at 55°C for 0 to 600 s. Reactions were then subjected to PCR amplification using the capillary-based LightCycler 1.5 with 30-second annealing/extension times.

Similar to previous results,[18] for multiple concentrations of pure SARS-CoV-2 RNA, efficient RT was observed within 30 seconds (Figure 1A). However, extraction-free positive patient samples required between 5 and 10

minutes for efficient RT (Figure 1, B and C). Next, the effect of increasing RT enzyme concentration on the time required for efficient RT was tested using both pure SARS-CoV-2 RNA as well as extraction-free positive patient samples. Higher RT enzyme concentrations further reduced the time required to perform efficient RT (Figure 1, D and E). However, important differences were observed when using extraction-free positive patient samples for the optimal time and RT enzyme concentrations required for efficient RT-qPCR (Figure 1E). With 1× RT enzyme concentration, >5 minutes were required for efficient RT-qPCR when using extraction-free patient samples; however, when 4× RT enzyme was used, this time could be reduced to 2 minutes for efficient RT-qPCR. These results demonstrate important differential kinetic limits for RT when using purified RNA versus extraction-free patient samples.

LightCycler PCR Cycle Time Optimization

An optimal PCR protocol was determined based on previous studies showing an inhibitory effect of extraction-free sample preparation on the PCR phase of SARS-CoV-2 RT-qPCR. Most commercial RT-PCR assays for SARS-CoV-2 have an annealing/extension time of ≥30 seconds per cycle. Annealing/extensions times of <0.5 seconds per cycle for short PCR products can result in efficient and specific amplification.[19,21] However, the previous studies using extreme PCR were conducted on purified DNA samples. Additionally, the CDC SARS-CoV-2 assay chosen uses hydrolysis probe-based detection, whereas extreme PCR uses intercalating dye chemistry for detection. Purified RNA or extraction-free positive patient samples were used to test for the optimal time required for this assay. Samples were reverse transcribed for 10 minutes prior to thermocycling.

Surprisingly, there was only a quantification cycle (Cq) difference of approximately 1 cycle for purified SARS-CoV-2 RNA when the annealing/extension step was set for 0 seconds versus 30 seconds, suggesting efficient PCR can be done with very rapid annealing/extension times (Figure 2A). However, extraction-free positive patient samples required a 10-second or greater annealing/elongation step for efficient PCR (Figure 2B), and this time could not be reduced by increasing Taq polymerase concentrations in the reaction (Figure 2, C and D). Furthermore, reactions with increased Taq polymerase concentrations showed increased Cq values at very short annealing/extension times of 0, 2, and 5 seconds for extraction-free positive patient samples, but little difference in Cq values for purified RNA. This suggests that additional annealing/extension time, and not Taq polymerase activity, is important for extraction-free

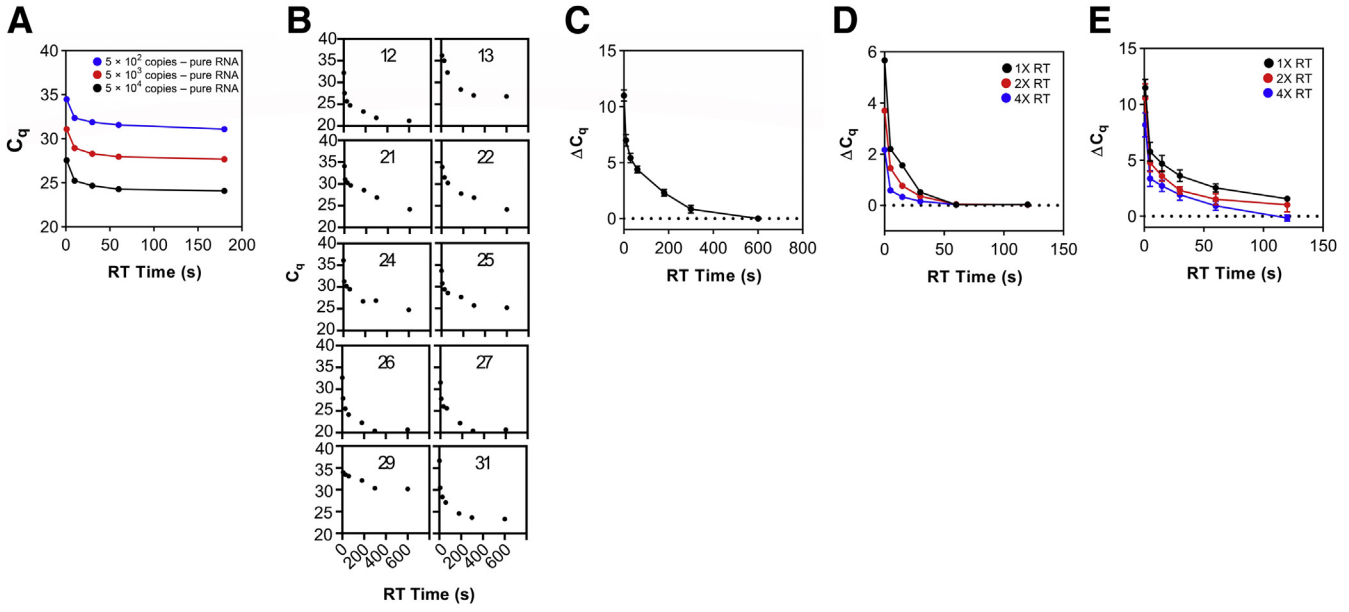

Figure 1 LightCycler reverse transcription optimization. **A:** Quantitative RT-PCR (RT-qPCR) was performed with various copy numbers of SARS-CoV-2 genomic RNA in water with a 30-second annealing/extension PCR step immediately after reverse transcription (RT) with 1× RT enzyme concentration for the indicated amounts of time. **B:** Representative extraction-free SARS-CoV-2—positive patient samples had RT performed for the indicated times using 1× RT enzyme concentration followed by PCR with a 30-second annealing/extension step. Quantification cycle (Cq) values decrease with increasing RT time. **C:** Summative data demonstrating average ΔCq for patient samples from **panel B**. **D:** Using 5 × 10^3 copies of SARS-CoV-2 genomic RNA in water, concentrations of RT enzyme were varied, and RT was performed for the indicated times prior to PCR with a 30-second annealing/extension step. The difference in quantification cycle (ΔCq) is relative to the longest time point Cq value. **E:** Using extraction-free SARS-CoV-2-positive patient samples, concentrations of RT enzyme were varied, and RT performed for the indicated times prior to PCR with a 30-second annealing/extension step. The difference in quantification cycle (ΔCq) is relative to the longest time point Cq value. Error bars represent SEM (**C** and **E**). $n = 11$ patient samples (**B**); $n = 3$ averaged technical replicates (**D**); $n = 3$ for each data point (**D**).

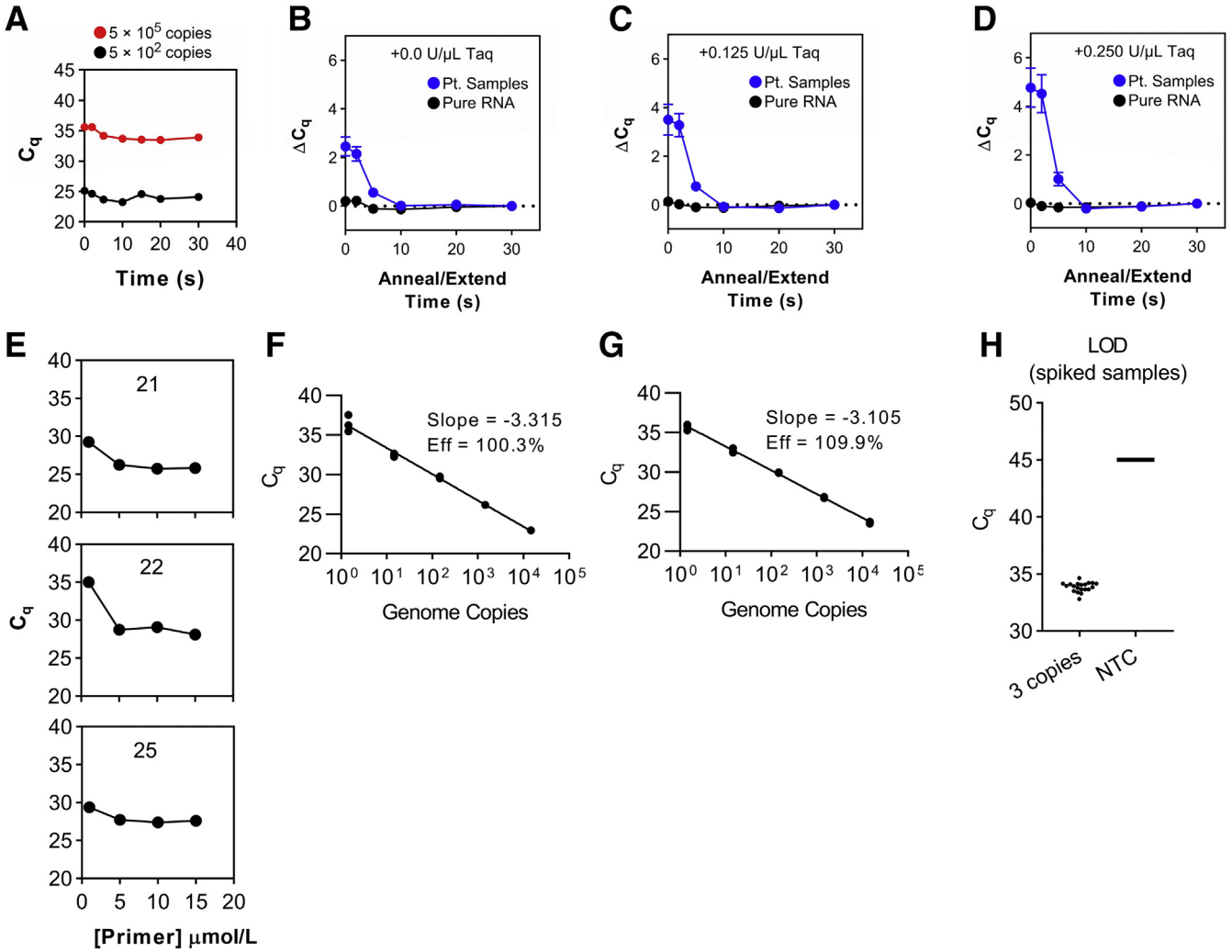

Figure 2 **A**—**D:** LightCycler PCR cycle time optimization. **A**—**D:** Quantitative RT-PCR (RT-qPCR) was performed with indicated copies of SARS-CoV-2 genomic RNA, a 10-minute reverse transcription (RT) step with 1× RT enzyme concentration and increasing times for the annealing/extension PCR step (**A**). A total of 5 × 10^3 copies of SARS-CoV-2 genomic RNA was spiked into saline or extraction-free SARS-CoV-2—positive patient samples and were reverse transcribed for 10 minutes with 1× RT enzyme concentration followed by PCR with increasing annealing/extension times with no additional Taq (**B**), 0.125 U/μL additional Taq (**C**), or 0.250 U/μL additional Taq (**D**). The difference in quantification cycle (ΔCq) is relative to the longest time point Cq value. For extraction-free SARS-CoV-2—positive patient samples, annealing/extension times of 10 to 30 seconds were optimal. **E:** Representative extraction-free SARS-CoV-2—positive patient samples were reverse transcribed for 10 minutes with 1× RT enzyme concentration followed by PCR with a 10-second annealing/extension step and increasing primer concentrations and a constant probe concentration of 250 nmol/L. **F:** Efficiency of extraction-free LightCycler assay with 5-minute reverse transcription and PCR cycles with a 10-second annealing/extension step using SARS-CoV-2 genomic RNA spiked into water. **G:** Efficiency of extraction-free LightCycler assay with 5-minute reverse transcription and PCR cycles with a 10-second annealing/extension step using SARS-CoV-2 genomic RNA spiked into uninfected control NP swab diluent. **H:** Low-copy RNA detection for the extraction-free LightCycler assay with 5-minute reverse transcription and PCR cycles with a 10-second annealing/extension step using SARS-CoV-2 genomic RNA spiked into uninfected control NP swab diluent. Twenty of 20 reactions containing three copies of SARS-CoV-2 genomic RNA were detected. Error bars represent SEM (**B**—**D**). $n = 4$ for each data point for patient samples (**B**—**D**); $n = 3$ averaged technical replicates for pure RNA for each data point (**B**—**D**). EFF, efficiency; NP, nasopharyngeal; Pt, patient.

detection of SARS-CoV-2 RNA. Using PCR cycles with a 10-second annealing/extension time, the impact of primer concentrations on PCR efficiency was examined using representative extraction-free positive patient samples. At primer concentrations two to four times higher (1 μmol/L) than those used in typical plate-based PCR assays (0.25 to 0.5 μmol/L), Cq values were approximately two to seven cycles higher compared with reactions containing five times higher primer concentrations (5 μmol/L), and further increases in primer concentration above 5 μmol/L showed little benefit (Figure 2E).

Using a 10-second annealing/elongation step, the reaction efficiency was examined using pure RNA as well as RNA spiked into diluent from an uninfected control NP swab. Both the pure RNA and spiked NP sample had efficiencies of 100.3% and 109.9%, respectively (Figure 2, F and G). Additionally, 20 of 20 reactions with three copies/reaction tested positive in spiked NP samples, suggesting adequate

detection of 2000 copies/mL when dry NP swabbing is performed for testing (Figure 2H).

Extraction-Free LightCycler SARS CoV-2 Virus Detection

Based on the current optimization studies, an RT-qPCR protocol that consisted of a 5-minute RT step at 55°C followed by a 10-second denaturation step at 95°C was adopted. This was then followed by 45 cycles of PCR with a 10-second annealing/elongation at 63°C with a 0-second hold time at 95°C. Overall, this PCR protocol takes a total of 20 minutes (Figure 3A). Forty-one positive patient samples were tested using extraction-free testing. Sixteen of the 42 samples were originally run on the University of Washington SARS-CoV-2 real-time RT-qPCR assay, and the remaining 26 were run on Panther Aptima TMA. Forty of 41 samples tested positive using the current assay on the capillary-based LightCycler. For the 15 of 16 samples that had Cq values from the original testing, a significant correlation was seen ($P < 0.001$, $r^2 = 0.763$) (Figure 3B). No correlation between the current assay's Cq values and the relative luminescence units reported was observed for the 26

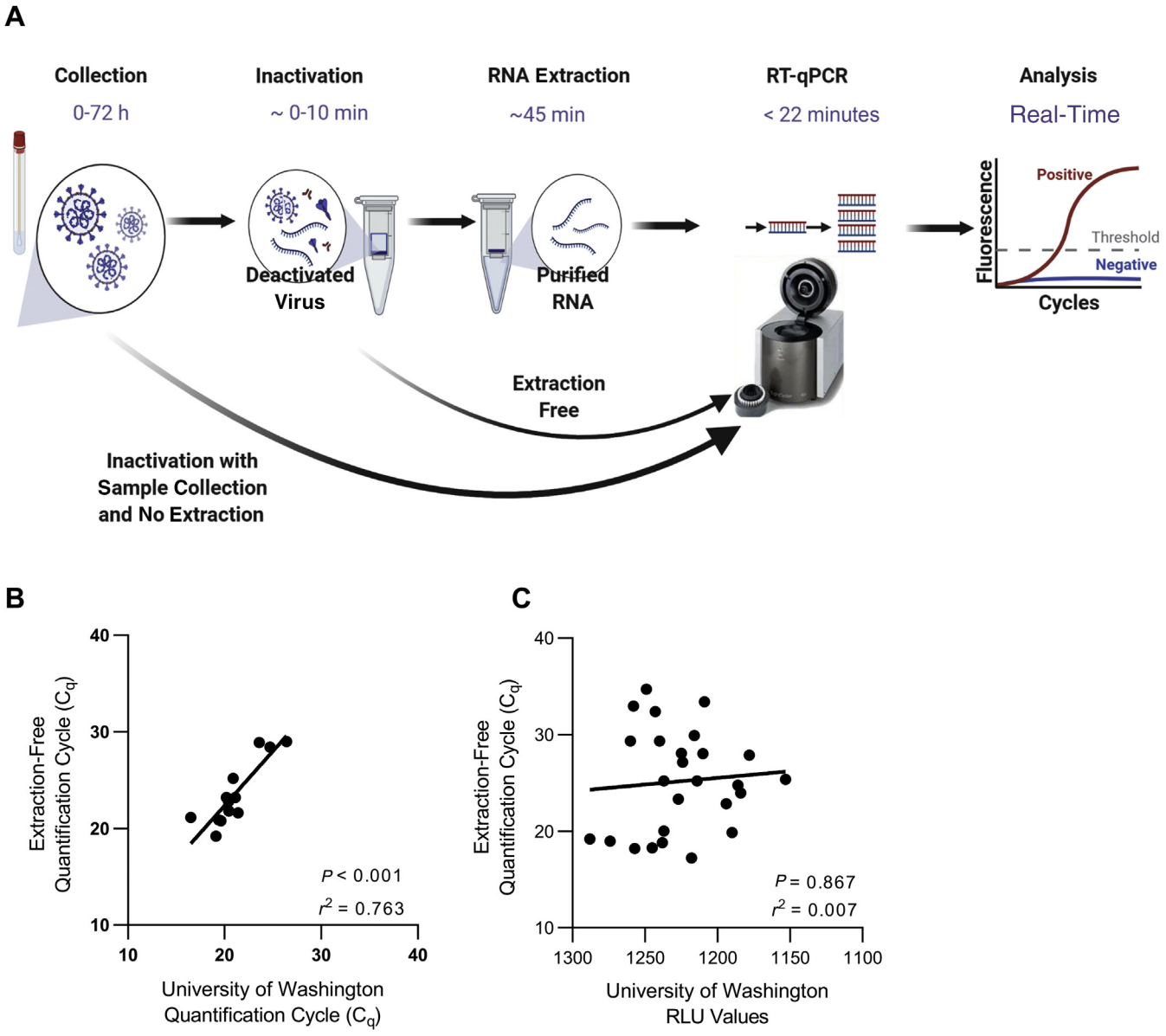

Figure 3 Extraction-free LightCycler assay validation. **A:** Schematic diagram of the extraction-free LightCycler protocol and typical times for collection and processing steps. **B:** Correlation analysis between quantification cycle (Cq) values from extraction-free LightCycler assay and Cq values obtained originally during testing at the University of Washington clinical virology laboratory. **C:** Correlation analysis between Cq values from extraction-free LightCycler assay and relative luminescence unit (RLU) values from the Panther Aptima TMA assay conducted on the original samples at the University of Washington clinical virology laboratory. Tm, melting temperature.

samples originally run on the Panther Aptima TMA platform ($P = 0.867$, $r^2 = 0.007$) (Figure 3C). This lack of correlation is expected, because the Panther Aptima TMA platform is an endpoint assay unlike traditional cycle threshold–based assays. For the one discordant sample, which did not test positive with the current protocol, the original Cq values on the University of Washington SARS-CoV-2 real-time RT-qPCR assay platform were 37.1 for one replicate and not detected for the other replicate. Such a high threshold cycle value with a negative result for the other replicate using RNA-extraction–based methods suggests a very low copy number that may be beyond the level of sensitivity of the current assay. For negative patient samples, the current protocol was 100% concordant (40/40; data not shown).

Extreme RT-PCR SARS-CoV-2 Virus Detection

Although the LightCycler protocol allowed for successful detection of SARS-Cov-2 in 20 minutes using extraction-free methodologies, the next set of experiments were designed to test whether the overall time for testing could be decreased using RNA extraction followed by extreme RT-PCR (Figure 4A). A different primer set targeting the nucleocapsid gene of SARS-CoV-2 was utilized for extreme RT-PCR. Successful amplification was measured by high-resolution melting curve analysis of the reaction product. Using a 30-second RT step at 60°C followed by 40 PCR cycles (approximately 1.4 seconds/cycle), purified SARS-CoV-2 genomic RNA was successfully amplified with detection down to four copies/reaction (Figure 4B). Additionally, amplification of 58 copies of SARS-CoV-2 genomic RNA spiked into NP swab diluent from a healthy, uninfected control was observed (Figure 4C). However, extraction-free SARS-CoV-2–positive patient samples were not successfully amplified (data not shown), likely due to both RT and PCR inhibition observed in these samples (Figures 1 and 2). Overall, extreme RT-PCR followed by high-resolution melting required <3 minutes to complete.

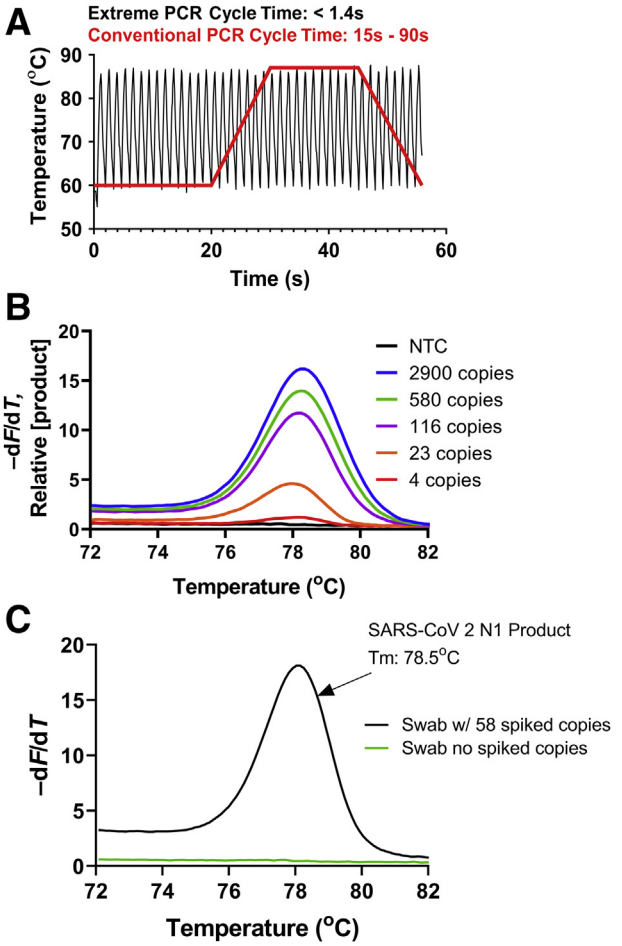

Figure 4 Extreme RT-PCR SARS-CoV-2 assay. **A:** 40 cycles of extreme PCR (**black**), completed in <60 seconds, are compared with a single traditional PCR cycle (**red**) in which there are long holds at temperatures and slow ramp-rate changes between temperatures. **B:** Extreme RT-PCR was performed on decreasing copies of SARS-CoV-2 genomic RNA spiked into water. Samples were amplified using a 30-second reverse transcription (RT) step at 60°C, followed by 40 cycles of PCR, with both completed in <90 seconds. High-resolution melting curve analysis was used to detect extreme RT-PCR amplification products. Using a constant excitation power during the melting, the height of the melting curve peaks is a good approximation for final product concentration. **C:** Extreme RT-PCR was performed on 58 copies of SARS-CoV-2 genomic RNA spiked into uninfected control NP swab diluent. The samples were amplified and analyzed as described in **panel B**. Melting curves are displayed as a derivative plot after exponential background removal and normalization. The *y* axis label, abbreviated as $-dF/dT$, is the negative first derivative of fluorescence with respect to temperature. NP, nasopharyngeal; NTC, no template control.

Discussion

The COVID-19 pandemic has made it abundantly clear that time to results for molecular diagnostic testing for pathogens is very important, not only in making clinical decisions, but also for identifying individuals that should quarantine to prevent further community transmission. Additionally, significant resources and time are required to keep patients isolated in emergency departments and hospitals until results from pathogen testing come back, which may take upto 2 days or more. However, it has also become clear that the sensitivity of an assay cannot be traded for speed, because false-negative results can have detrimental and life-threatening impacts to patients, healthcare workers, and the community.

Although many new advances in molecular diagnostic testing have been developed in response to the COVID-19 pandemic, shortages in rapid testing supplies have remained. Several groups have shown the feasibility of extraction-free RT-PCR for SARS-CoV-2. This methodology not only decreases time requirements of testing but also allows for more economical testing. In addition to the time requirements of RNA isolation, typical plate-based thermal cycler RT-PCR reactions take >60 minutes, putting the

entire process of RNA isolation and RT-PCR at several hours.

The kinetic constraints of extraction-free RT-PCR for SARS-CoV-2 RNA detection were examined to facilitate the creation of an economical and rapid molecular diagnostic test for COVID-19. Although extreme and rapid PCR are typically conducted without a probe,[19,22] the probe-based methodology was used for increased specificity. Additionally, the LightCycler (Roche) RT-PCR instrument was used for thermocycling because it is able to rapidly thermocycler, allowing for better optimization studies.[23] However, this methodology was also applied to a plate-based instrument (QuantStudio 3; Thermo Fisher Scientific, Waltham, MA) with similar results, albeit with a longer overall time of approximately 30 minutes due to slower temperature ramp rates (data not shown), showing the broader adaptability of this methodology. Additionally, QuantStudio 3 was used to multiplex the CDC N1 primer set with the CDC *RPP30* primer set with results concordant to the current LightCycler assay (data not shown). These results suggest that both the RT (Figure 1) and PCR (Figure 2) steps are inhibited during extraction-free RT-PCR. Interestingly, differential inhibition between patient samples was observed with a more striking difference in inhibition between uninfected spiked samples and positive samples for both RT and PCR. This suggests a possibility of increased inhibition with increased inflammation/mucus during infection. This further confirms that optimization studies require the use of samples from infected patients rather than uninfected spiked samples.

The current data (Figures 1, B-D) shows that increasing Taq concentration in extraction-free RT-PCR does not decrease the required anneal/extension time, suggesting that the inhibition is not polymerase concentration−dependent, but rather the inhibition more likely affects the processivity rate of the Taq polymerase. Additional experiments would be needed to parse out whether the inhibition seen in extraction-free RT-PCR primarily affects the polymerase activity or $5' \rightarrow 3'$ exonuclease activities of Taq. An increase in primer concentration (5 μmol/L) was required for the rapid thermocycling ($\leq$10 seconds) in this assay. Although the primer concentration was increased approximately 10-fold above traditional primer concentrations, the assay required only a modest twofold increase in probe concentration (250 nmol/L). Future studies will be needed to determine whether optimization of primer and probe concentrations can additionally increase the speed of this protocol.

Using a total of 20% sample in the reaction, extraction-free RT-PCR requires approximately 5 to 10 minutes for efficient RT and 10-second annealing/elongation per cycle for PCR, which results in a total time of 20 minutes for a 45-cycle reaction using a LightCycler 1.5 (Roche). However, using isolated RNA, RT-PCR can be shortened to <30 seconds for RT and <60 seconds for 45 cycles of PCR for a total reaction time of approximately 90 seconds. These results suggest that both methods can allow for rapid testing, but the limit for extraction-free samples seems to be approximately 20 minutes. The difference in time requirements for these two assays (approximately 18 minutes) suggests that if a rapid RNA isolation step is implemented, the time required may be decreased even further. Pairing together microfluidic and rapid thermocycling platforms may allow for rapid RNA isolation and extreme RT-PCR thermocycling for an even faster result. Another potential way to decrease inhibition would be to use a smaller amount of patient sample (<20%); however, this may decrease overall sensitivity of the assay. Additionally, it may be possible to increase the speed of our extraction-free protocol using polymerases and/or reverse transcriptases that are known to have higher inhibitor resistance.[24,25]

Additional time could be saved by collecting samples directly into a low-complexity buffer (saline or molecular grade water) containing Triton X-100. Future clinical studies examining the differential inhibition of NP, oropharyngeal, and sputum samples are warranted to further refine optimal sample collection for the current protocol and other extraction-free methods. Although this study did not directly examine the role of different detergents and/or heating strategies for extraction-free RT-PCR, optimization with Tween-20, NP-40, or other detergents in combination with a rapid heating step may further allow for more rapid RT and PCR. The current results showing successful amplification of 58 copies of SARS-CoV-2 using extreme RT-PCR, but not in the presence of matrix from patients positive for SARS-CoV-2, suggests a different and/or increased inhibitory mechanism in these patients versus healthy controls. Future studies will be needed to elucidate this mechanism as well as ways to address this inhibition for extraction-free extreme RT-PCR to be feasible.

Although testing of the protocol developed in these studies was limited to the use of CDC N1 primers and extreme RT-PCR primers residing in the N gene of SARS-CoV-2, these results lay the groundwork for a rapid and economical molecular diagnostic assay that still maintains the sensitivity and specificity required for accurate diagnoses. This study demonstrates the feasibility of swab to result RT-PCR using an *in vitro* diagnostic-approved LightCycler (Roche) paired with commercially available RT-PCR reagents. Both methods show sensitivity and specificity required for screening patients for SARS-CoV-2.

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