Evaluation of a High-Definition PCR Assay for the Detection of SARS-CoV-2 in Extracted and Nonextracted Respiratory Specimens Collected in Various Transport Media

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ABSTRACT

Objectives: *We conducted an analytic and clinical comparison of a novel high-definition polymerase chain reaction PCR (HDPCR) assay to traditional real-time PCR (RT-PCR) for the detection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in upper respiratory specimens.*

Methods: *Analytic performance of RT-PCR, HDPCR, and extraction-free HDPCR was established through replicate testing of a serially diluted clinical specimen containing SARS-CoV-2. A clinical comparison of all 3 assays was conducted using 351 prospectively collected upper respiratory swab specimens obtained from symptomatic and asymptomatic individuals collected in various transport media.*

Results: *RT-PCR and HDPCR assays using extracted nucleic acid demonstrated similar analytic limits of detection (LoD) and clinical performance, with 100% positive and negative agreement. Extraction-free HDPCR demonstrated a 1.5 to 2.0 log$_{10}$ increase in LoD based on cycle threshold values. However, clinical performance of extraction-free HDPCR remained high, demonstrating 97.8% positive and 99.6% negative agreement with RT-PCR. An overall increase in "invalid" and "presumptive" results was observed when using the extraction-free method, but this was highly variable based on transport medium used.*

Key Points

- HDPCR demonstrated similar performance to traditional RT-PCR for the detection of SARS-CoV-2.
- Use of an extraction-free HDPCR protocol demonstrated similar clinical performance to RT-PCR and HDPCR using extracted template while reducing hands-on time and cost.
- The incidence of "invalid" and "presumptive" SARS-CoV-2 results obtained using extraction-free HDPCR differed by transport medium used.

Conclusions: *HDPCR performs similar to RT-PCR for the detection of SARS-CoV-2. The use of an extraction-free HDPCR protocol maintained high clinical performance despite reduced analytic LoD, with the benefit of reduced hands-on time and cost of reagents associated with nucleic acid extraction.*

Identification of individuals infected with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), symptomatically or asymptomatically, is essential for appropriate management of the acutely ill and for enabling appropriate infection prevention, quarantine, and contact tracing for those that may be infectious. Nucleic acid amplification tests (NAATs) are considered the gold standard method for detection of SARS-CoV-2 viral RNA in clinical specimens. Currently, over 200 NAATs have received emergency use authorization (EUA) status from the US Food and Drug Administration.[1] These tests can be broadly classified as on-demand sample-to-answer cartridge-based

tests (eg, Xpert Xpress SARS-CoV-2, BioFire RP2.1, ID NOW COVID-19), high-throughput sample-to-answer tests (eg, Aptima SARS-CoV-2, cobas SARS, Alinity m SARS-CoV-2), and high-complexity manual batch-based tests (eg, TaqPath COVID-19, Quidel Lyra SARS-CoV-2, CDC 2019 nCoV RT-PCR). Clinical laboratories frequently offer each of these test types to accommodate the needs of different patient populations: acutely ill emergency room or inpatient, preadmission or presurgical screening, symptomatic ambulatory, and asymptomatic exposure or population screening.

Manual batch-based tests consist of polymerase chain reaction (PCR) reagents that can be utilized on various existing open-platform real-time PCR (RT-PCR) thermocyclers present in most clinical laboratories. These thermocyclers are often capable of accommodating 96 or 384-well PCR plates, and when used in conjunction with SARS-CoV-2 RT-PCR reagents have the potential to provide the highest throughput and lowest cost per specimen among laboratory-based NAATs. However, these tests require extraction of nucleic acids from specimens prior to RT-PCR amplification and detection. In addition to hands-on time required to manually set up extraction and RT-PCR plates, the additional reagents and disposables required increase the total cost per result and present increased potential for supply chain gaps. Further, many automated nucleic acid extraction platforms can process only 16 to 24 specimens per batch, which can lead to workflow bottlenecks and extended turnaround time. Given these barriers, adaptation of batch-based tests using a direct or extraction-free protocol would be desirable and provide several potential benefits to cost, hands-on time, turnaround time, throughput, and reduced risk of downtime due to supply chain shortages.

Prior studies have examined an extraction-free approach using either chemical or heat-based lysis steps and noted decreases in analytic limit of detection (LoD) as well as clinical sensitivity.[2-4] Differences in sensitivity between extracted and nonextracted specimens can be attributed to multiple variables, including (1) volume and type of transport medium put into the direct RT-PCR reaction, (2) genomic target and length of amplicon, (3) lack of specimen concentration in nonextracted specimens, and (4) stability of the polymerase enzyme utilized. These factors resulted in median increases of up to 6.7 cycle threshold (CT), which is equivalent to an approximately 2 $\log_{10}$ decrease in analytic sensitivity. Importantly, in these studies the clinical sensitivity also fell to 72% to 81% when compared to extracted specimens, with false-negative results weighted toward those specimens with high CT values.[2-4] While these data involve laboratory-developed

protocols, the decreased analytic sensitivity associated with extraction-free protocols is also observed when comparing tests that have received EUA. Specifically, the Lyra SARS-CoV-2 Direct assay (Quidel) demonstrates an approximately 3 $\log_{10}$ decrease in analytic LoD when compared to the Lyra SARS CoV-2 assay that uses extracted viral RNA as template.[5]

The EUA high-definition PCR (HDPCR) SARS-CoV-2 assay (ChromaCode) is a multiplexed molecular test that targets 2 regions of the SARS-CoV-2 nucleocapsid gene (N1 and N2), as well as the human RNase P gene (RP) as an internal control, in a single well. HDPCR utilizes standard RT-PCR instrumentation and well-established hydrolysis probe chemistry; however, in addition to differentiation of each target based on unique fluorophores, HDPCR employs a limiting probe design. The use of limited probe does not impact detection of a specific target (ie, CT value); however, the maximal fluorescence signal (ie, amplitude) reached during plateau phase of PCR can be modulated based on the amount of probe included in the reaction. This design enables differentiation of multiple unique targets using the same fluorophore based on the endpoint fluorescent signal or plateau associated with each probe. In addition to differentiation of multiple targets in a single fluorescent channel, HDPCR may also increase specificity of target detection. This HDPCR technology has successfully been applied to multiplex molecular tests targeting multiple respiratory viruses in nasopharyngeal specimens as well as multiple tickborne pathogens in whole-blood samples with high sensitivity and specificity.[6-8]

The primary aim of our study was to provide an analytic and clinical performance comparison between a traditional RT-PCR assay, TaqPath COVID-19 Combo Kit (ThermoFisher), and the HDPCR SARS-CoV-2 Assay (ChromaCode). Further, we examined the feasibility, performance, and potential impact of a research use only extraction-free protocol using the HDPCR SARS-CoV-2 reagents.

Materials and Methods

Prospective Clinical Specimen Enrollment

A total of 351 remnant specimens obtained from both symptomatic and asymptomatic patients with a clinical test order for SARS-CoV-2 NAAT were prospectively collected and enrolled at Froedtert and the Medical College of Wisconsin, and Wisconsin Diagnostic Laboratories in Milwaukee, WI. All

specimens were collected between September 1, 2020 and September 15, 2020, and were stored at 4°C for up to 72 hours prior to testing. Specimens included dual nasopharyngeal and oropharyngeal swab collection devices utilizing several different transport media (universal transport medium [UTM], Copan; M4-RT, Remel; viral transport medium [VTM], Hardy; smart transport medium [STM], MedSchenker; ESwab, Copan; VTM, Gentueri; VTM, Wisconsin Veterinary Diagnostic Laboratory; VTM, laboratory developed; saline, manufacturer unknown).

TaqPath COVID-19 Assay

A 200-µL aliquot of each specimen was used as input for automated nucleic acid extraction. Extraction was conducted using the MagMAX Viral/Pathogen reagents (ThermoFisher) and KingFisher Flex Magnetic Particle Processor (ThermoFisher) in accordance with the TaqPath coronavirus disease 2019 (COVID-19) Combo Kit instructions for use (revision F.0, July 15, 2020). Purified nucleic acid was eluted in a final volume of 50 µL buffer, and was used as template for both TaqPath COVID-19 and HDPCR SARS-CoV-2 assays.

The TaqPath COVID-19 assay was set up in accordance with the manufacturer's instruction for use. Briefly, TaqPath reagents were thawed and combined to make a fresh mastermix. A 10-µL volume of nucleic acid extract was added to a 15-µL volume of mastermix in each well of a 96-well PCR plate. Each plate also contained a positive and negative control. Thermocycling was conducted using the 7500 Fast Dx Real-Time PCR instrument (Applied Biosystems) in accordance with TaqPath COVID-19 instructions for use for a total of 40 cycles. Upon completion, raw RT-PCR data were transferred to a second computer containing the COVID-19 Interpretive Software for analysis (version 1.3). Detection of any 2 of 3 assay targets (ORF1ab, N gene, S gene) with CT ≤ 37 was considered "positive," detection of a single target was considered "inconclusive," and failure of the MS2 internal control was considered "invalid."

HDPCR SARS-CoV-2 Assay

The HDPCR SARS-CoV-2 assay was set up in accordance with the manufacturer's instruction for use. Briefly, HDPCR reagents were thawed and combined to make a fresh mastermix. A 5-µL volume of nucleic acid extract (above) was added to a 15-µL volume of mastermix in each well of a 96-well PCR plate. Each plate

also contained a positive control, a negative control, and 5 calibrators to enable calibration of the expected fluorescent signal strength of each target. Thermocycling was conducted using the 7500 Fast Dx Real-Time PCR instrument (Applied Biosystems) in accordance with HDPCR SARS-CoV-2 instructions for use for a total of 55 cycles. Upon completion, raw RT-PCR data were uploaded to the ChromaCode Cloud Interpretive Software for analysis. Detection of both SARS-CoV-2 assay targets (N1, N2) with CT ≤ 55 and fluorescent signal ≥ 50% of calibrator signal was considered "positive," detection of a single target (N1 or N2) was considered "presumptive," and failure to detect the RP internal control was considered "invalid."

HDPCR SARS-CoV-2 Assay, Extraction-Free Protocol

The extraction-free HDPCR protocol was very similar to the EUA protocol with the following modifications: (1) in lieu of extracted nucleic acid, 5 µL of raw specimen (ie, swab in transport medium) was added directly to 15 µL of HDPCR mastermix in a 96-well PCR plate; and (2) the thermocycling protocol was modified to include an initial 15-minute incubation at 58°C prior to initiating the 15-minute reverse transcription at 50°C and thermocycling as indicated in the manufacturer's instructions for use. A temperature of 58°C was chosen for the preincubation/lysis step based on optimization studies (unpublished data) to strike a balance between effective viral lysis and preservation of the heat-labile reverse transcriptase enzyme, which is stable to 62°C. All other steps were performed exactly as in the EUA instructions.

Limit of Detection Study

A single positive clinical specimen collected in M4-RT was selected for use in a comparative LoD study between the TaqPath COVID-19, HDPCR SARS-CoV-2, and extraction-free HDPCR protocols. This specimen was reported as "positive" by cobas SARS-COV-2 with CT values of 29.3 (ORF1) and 30.4 (E-gene). Five serial 10-fold dilutions (10^{-1} to 10^{-5}) of the specimen were made in fresh M4-RT medium. Two parallel nucleic acid extractions were conducted on each dilution, and individual 50-µL extracts were combined for a total volume of 100 µL per dilution. A total of 15 replicate HDPCR SARS-CoV-2 RT-PCR reactions (5-µL extract each) and 10 replicate TaqPath COVID-19 RT-PCR reactions (10-µL extract each) were tested for each of the 5 dilutions as well as the original undiluted specimen. In parallel, 15 replicates of each specimen were tested using the HDPCR

SARS-CoV-2 extraction-free protocol using 5 μL of the direct, unextracted specimen as template. The number of replicates reported as "detected," "inconclusive/presumptive" (ie, single target detected), and "negative" were recorded in addition to CT values.

Time in Motion Study

A standard timer was used to record the time necessary for a technologist to manually prepare a 96-well deep plate for nucleic acid extraction. This included addition of proteinase K to each well using a repeat pipettor, followed by sequential addition of MS2 internal control and binding buffer containing magnetic beads each using a multichannel pipettor, and finally addition of each individual specimen using a single-channel standard pipette. Similarly, the time necessary to manually set up a 96-well PCR plate was recorded. This included addition of mastermix using a repeater pipette, followed by addition of either extracted nucleic acid or raw specimen as template using a standard pipette. The average hands-on time for preparation of extraction and PCR plates was recorded across 14 independent plates each, as conducted by 4 different technologists and across 4 different days. The KingFisher (ThermoFisher) extraction runtime was a consistent 27 minutes and thermocycler run times were 65 minutes for TaqPath, 105 minutes for HDPCR, and 120 minutes for extraction-free HDPCR programs. Reagent prices were obtained from the manufacturers' websites accessed January 6, 2021.

Statistical Analysis

Standard equations in Excel (Microsoft) were used to conduct regression analysis and to calculate mean and standard deviation of the data sets. Positive and negative percent agreement and 95% confidence intervals were calculated using VassarStats website for statistical computation http://vassarstats.net/.

Results

Limit of Detection Comparison

Replicate tests were performed on a serially diluted positive clinical specimen using TaqPath COVID-19, HDPCR SARS-CoV-2, and extraction-free HDPCR SARS-CoV-2 assays **Table 1**. Detection of the internal control was required for a result of "negative" for SARS-CoV-2. The internal control for the TaqPath assay consists of MS2 phage template exogenously added to each specimen prior to extraction, and was detected in all replicates at all dilutions. The internal control for the HDPCR assays is the human RP gene, which is endogenous to the specimen. As expected, this internal control was diluted along with the clinical specimen and became undetectable in 1 of 5 (20%) replicates at the 10^{-4} dilution for the extracted HDPCR; as well as 1 of 15 (6.7%) at 10^{-2} and 8 of 15 (53.3%) at 10^{-3} dilution for extraction-free HDPCR replicates. Differences in internal control detection rate between

■Table 1■

Comparison of the Limit of Detection Between TaqPath COVID-19, HDPCR SARS-CoV-2, and HDPCR SARS-CoV-2 Extraction-Free Assays[a]

Assay	Dilution				
	10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}
TaqPath COVID-19					
IC detected	**10/10 (100)**	**10/10 (100)**	**10/10 (100)**	**10/10 (100)**	**5/5 (100)**
Positive	**10/10 (100)**	**10/10 (100)**	**10/10 (100)**	3/10 (30.0)	0/5 (0.0)
Inconclusive[b]	0/10 (0.0)	0/10 (0.0)	0/10 (0.0)	7/10 (70.0)	0/5 (0.0)
Total detected[c]	**10/10 (100)**	**10/10 (100)**	**10/10 (100)**	**10/10 (100)**	0/5 (0.0)
HDPCR SARS-CoV-2					
IC detected	**15/15 (100)**	**15/15 (100)**	**15/15 (100)**	**15/15 (100)**	1/5 (20.0)
Positive	**15/15 (100)**	**15/15 (100)**	**15/15 (100)**	11/15 (73.3)	0/1 (0.0)
Presumptive[b]	0/15 (0.0)	0/15 (0.0)	0/15 (0.0)	2/15 (13.3)	0/1 (0.0)
Total detected	**15/15 (100)**	**15/15 (100)**	**15/15 (100)**	13/15 (86.7)	0/1 (0.0)
Extraction-free HDPCR SARS-CoV-2					
IC detected	**15/15 (100)**	**15/15 (100)**	14/15 (93.3)	7/15 (46.7)	0/5 (0.0)
Positive	**15/15 (100)**	13/15 (86.7)	3/14 (21.4)	0/7 (0.0)	NA
Presumptive[b]	0/15 (0.0)	2/15 (13.3)	4/14 (28.6)	1/7 (14.3)	NA
Total detected	**15/15 (100)**	**15/15 (100)**	7/14 (50.0)	1/7 (14.3)	NA

COVID-19, coronavirus disease 2019; HDPCR, high-definition polymerase chain reaction; IC, internal control; NA, not applicable; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.
[a]Data are No. detected/total No. (%). Bold font indicates detection greater than 95.0% of replicates.
[b]One of 2 SARS-CoV-2 targets detected.
[c]Any number of SARS-CoV-2 targets detected, ie, positive or presumptive/inconclusive.

extracted and extraction-free HDPCR dilutions can be partly attributed to a 4-fold concentration of the specimen during nucleic acid extraction, resulting in 4-fold more target being added to the final PCR reaction for extracted specimens.

Results of TaqPath and HDPCR assays using extracted nucleic acid template demonstrated a similar analytic LoD, with 100% of replicates being reported as "positive" at 10^0, 10^{-1}, and 10^{-2} dilutions. At 10^{-3} TaqPath reported just 3 of 10 (30.0%) replicates as "positive" and the remaining 7 of 10 (70.0%) as "inconclusive," meaning only 1 of 2 SARS-CoV-2 targets were detected. In contrast, at 10^{-3} HDPCR reported 11 of 15 (73.3%) replicates as "positive" and 2 of 15 (13.3%) as "presumptive," indicating detection of only 1 of 2 SARS-CoV-2 targets. Based on these data, TaqPath and HDPCR have a similar LoD when using extracted nucleic acid. However, at low viral concentrations, HDPCR reported a larger proportion of replicates as definitively "positive" (73.3% vs 30.0%, χ^2 $P = .024$) but a lower total proportion as "positive" or single-target "presumptive/inconclusive" (86.7% vs 100%, χ^2 $P = .698$).

The analytic LoD of the extraction-free HDPCR protocol was approximately 2 $\log_{10}$ higher than extracted methods, with 100% of replicates detected at 10^0, but only 13 of 15 (86.7%) detected at 10^{-1} and 3 of 14 (21.4%) detected at 10^{-2}. However, an additional 2 of 15 (13.3%) and 4 of 14 (28.6%) of replicates were reported as "presumptive" at 10^{-1} and 10^{-2}, respectively, raising the percentage of replicates with at least 1 target detected to 100% and 50%, respectively.

Linearity and Cycle Threshold of HDPCR and HDPCR Extraction-Free Protocols

To further investigate the difference in analytic LoD observed between the standard and extraction-free HDPCR protocols, we compared the CT values and linearity obtained for the diluted specimen replicates. The average CT value across all replicates was approximately

3.7 to 4.0 higher for N1, 4.1 to 5.5 higher for N2, and 3.4 to 6.0 higher for RP when comparing the extraction-free protocol to the EUA HDPCR assay **Table 2** and **Figure 1**. Assuming approximately 3.3 CT per 1 $\log_{10}$ difference in viral concentration, this equates to an approximate 1.3 $\log_{10}$ to 1.7 $\log_{10}$ difference between extracted and extraction-free protocols for SARS-CoV-2 N1 and N2 targets. A portion of this decrease is attributable to the 4-fold concentration during nucleic acid extraction, which results in more starting template in RT-PCR and HDPCR reactions using extracted template. Notably, the variance in recorded CT value (determined by standard deviation) was greater for the extraction-free protocol, and increased with increasing dilution of specimens in both standard and extraction-free protocols. When the average CT value of all replicates was plotted against the log dilution factor, N1, N2, and RP all demonstrated excellent linearity with line of regression (R^2) values of greater than 0.99 when using extracted nucleic acid template (Figure 1). The R^2 values obtained for the extraction-free protocol were also high, ranging from 0.97 to 0.98 for each of the assay targets.

Clinical Comparison of TaqPath COVID-19 and HDPCR SARS-CoV-2 Assays

Results of the HDPCR SARS-CoV-2 assay were compared to the TaqPath COVID-19 assay among 351 prospectively collected clinical specimens. Two of 351 (0.6%) specimens were reported as "inconclusive" by TaqPath and were excluded from analysis. Among the remaining 349 specimens there was 100% (50/50) positive agreement and 100% (298/298) negative agreement between the 2 assays with a single specimen (0.3%) reported as "invalid" by HDPCR due to failed detection of the RP internal control **Table 3**.

Clinical Comparison of HDPCR SARS-CoV-2 and HDPCR SARS-CoV-2 Extraction-Free Assays

Raw, unextracted specimen in transport medium (5 µL) obtained from the 349 specimens with a valid

Table 2

Difference in CT Values Between Standard HDPCR and HDPCR Extraction-Free Assays

Dilution	Target N1, Average CT (SD)			Target N2, Average CT (SD)			Target RP, Average CT (SD)		
	HDPCR	Extraction-Free	Δ	HDPCR	Extraction-Free	Δ	HDPCR	Extraction-Free	Δ
10^0	22.8 (0.1)	26.8 (0.7)	−4.0	24.1 (0.1)	29.0 (0.7)	−4.9	23.2 (0.3)	26.6 (0.4)	−3.4
10^{-1}	27.0 (0.2)	30.8 (1.9)	−3.8	28.3 (0.3)	32.4 (1.6)	−4.1	26.7 (0.4)	31.1 (0.5)	−4.4
10^{-2}	30.7 (0.4)	34.4 (1.0)	−3.7	32.0 (0.4)	37.5 (2.1)	−5.5	30.5 (0.6)	36.5 (1.0)	−6.0
10^{-3}	34.2 (0.9)	ND[a]	—	35.4 (0.5)	40.3[b]	−4.9	33.0 (0.5)	39.4 (2.6)	−6.4

CT, cycle threshold; HDPCR, high-definition polymerase chain reaction; N, nucleocapsid; RP, RNase P; SD, standard deviation.
[a]Target N1 was not detected (ND) in any of the replicates.
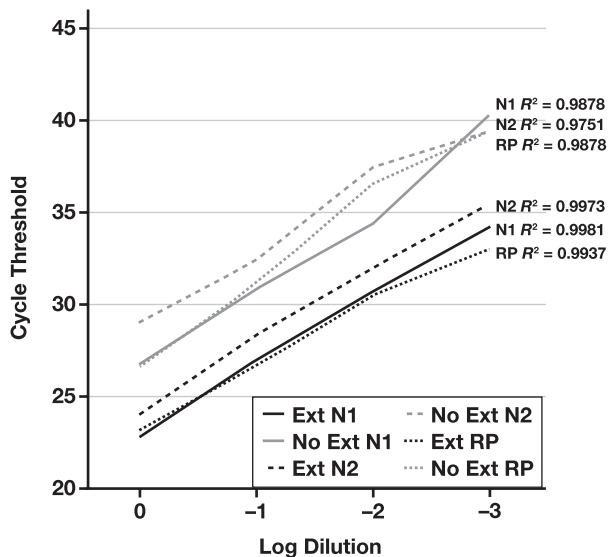[b]Target N2 was detected in a single replicate.

❚Figure 1❚ Linearity of HDPCR and HDPCR extraction-free protocols. CT values obtained from replicate tests of a serially diluted specimen were plotted to establish differences in average CT value between standard HDPCR using extracted nucleic acid template (Ext) and the extraction-free protocol (No Ext) using raw specimen as template. Values for each SARS-CoV-2 assay target (N1 and N2) as well as the internal control (RP) were reported and a regression analysis of the best fit line was calculated (R^2). CT, cycle threshold; HDPCR, high-definition polymerase chain reaction; N, nucleocapsid; RP, RNase P; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

TaqPath result was used as template for the extraction-free HDPCR assay. Use of raw specimen resulted in an increased incidence of "invalid" results (18/349, 5.1%) when compared to HDPCR using extracted nucleic acid template (1/349, 0.3%). This is likely the result of inhibitory substances in the raw specimens or transport medium that were removed during nucleic acid extraction. Invalid results were unevenly distributed across the 9 transport media used, ranging from 0% for Hardy VTM, laboratory developed VTM, ESwab, and saline, to 28.6% for MedSchenker STM (Table 3). Similarly, the incidence of single-target "presumptive" results increased from 0% (0/349) when using extracted nucleic acid template to 3.3% (11/331) when using raw specimen. The "presumptive" results were also unequally distributed across transport media, ranging from 0% for Copan VTM and WVDL VTM to 18.2% (6/33) for Gentueri VTM and 33.4% (1/3) for saline. Among the specimens reported as "presumptive," 6 of 11 (54.4%) were reported as "positive" by both HDPCR and TaqPath using extracted nucleic acid template, while the remaining 5 of 11 (45.6%) were reported as "negative" by both extracted assays. When each of

the 11 specimens were retested using a fresh 5 µL of raw specimen, 7 (63.6%) repeated as "presumptive," 2 (18.2%) repeated as "negative," and 1 each (9.1%) repeated as "positive" and "invalid."

When the 11 "presumptive" results were removed from analysis, the positive predictive value and negative predictive value of extraction-free HDPCR were 97.8% (44/45) and 99.6% (274/275), respectively, compared to TaqPath (Table 3). Both discordant results were observed in specimens collected using Gentueri VTM. The false-positive specimen had initial CT values of 29.3 (N1) and 30.8 (N2) using the extraction-free protocol, and repeat analysis resulted in a "presumptive" call with only N1 detected, CT 31.0. The false-negative specimen was also reported as negative upon repeat analysis, but was positive by both TaqPath and HDPCR using extracted nucleic acid template with CT values of 32.7 (N1) and 34.3 (N2).

Time-in-Motion and Utilization of Resources

The technologist time required to manually set up a 96-well extraction plate and subsequent 96-well PCR plate was established across 14 independent batches of respiratory swab specimens submitted for SARS-CoV-2 testing. Testing was completed by 4 different technologists across 4 different days. The average time to set up an extraction plate was 26.6 minutes (range, 23-30), inclusive of manual addition of all 3 extraction reagents and specimen to each well. The average time to set up a PCR plate was 21.5 minutes (range, 20-24), inclusive of addition of mastermix and template to each well. In addition, the automated extraction time was 27 minutes, and the thermocycling times were 65 minutes for TaqPath, 105 minutes for HDPCR, and 120 minutes for extraction-free HDPCR assays. Based on these data, utilization of the extraction-free HDPCR protocol reduces total hands-on time by approximately 55.3% (21.5 minutes vs 48.1 minutes) and total time to result by 21.4% (141.5 minutes vs 180.1 minutes) when compared to standard HDPCR. Total time to result is similar between extraction-free HDPCR and TaqPath (141.5 minutes vs 140.1 minutes) due to the longer thermocycling of the extraction-free protocol; however, elimination of the nucleic acid extraction step also significantly reduces reagent cost. The list price of extraction reagents used in this study equates to approximately $1.58/specimen or $152.40 per 96-well plate, in addition to approximately $15.00 in plastics (deep-well plate, pipette tips).

Discussion

Unprecedented demand for SARS-CoV-2 testing during the current pandemic has resulted in supply chain

Table 3

Clinical Performance of the HDPCR SARS-CoV-2 Assay

Media (Manufacturer)	TP	TN	FP	FN	Total	PPA, % (95% CI)	NPA, % (95% CI)	% Pres	% Inv
M4-RT (Remel)									
Extracted	17	83	0	0	100	100 (76-100)	100 (94-100)	0.0	1.0
Extraction-free	16	80	0	0	96	100 (74-100)	100 (94-100)	2.0	3.0
VTM (WVDL)									
Extracted	9	46	0	0	55	100 (63-100)	100 (90-100)	0.0	0.0
Extraction-free	9	42	0	0	51	100 (63-100)	100 (90-100)	0.0	7.3
VTM (Hardy)									
Extracted	5	45	0	0	50	100 (46-100)	100 (90-100)	0.0	0.0
Extraction-free	4	45	0	0	49	100 (40-100)	100 (90-100)	2.0	0.0
UTM (Copan)									
Extracted	10	31	0	0	41	100 (66-100)	100 (86-100)	0.0	0.0
Extraction-free	10	29	0	0	39	100 (66-100)	100 (85-100)	0.0	4.9
VTM (Gentueri)									
Extracted	3	31	0	0	34	100 (31-100)	100 (86-100)	0.0	0.0
Extraction-free	1	25	1[a]	1[b]	28	50.0 (3-97)	96.2 (78-99)	18.2	2.9
STM (MedSchenker)									
Extracted	3	25	0	0	28	100 (31-100)	100 (83-100)	0.0	0.0
Extraction-free	2	17	0	0	19	100 (20-100)	100 (77-100)	5.0	28.6
ESwab (Copan)									
Extracted	1	15	0	0	16	100 (55-100)	100 (75-100)	0.0	0.0
Extraction-free	0	15	0	0	15	NA	100 (75-100)	6.3	0.0
VTM (laboratory developed)									
Extracted	2	7	0	0	9	100 (20-100)	100 (56-100)	0.0	0.0
Extraction-free	2	7	0	0	9	100 (20-100)	100 (56-100)	0.0	0.0
Saline									
Extracted	0	3	0	0	3	NA	100 (31-100)	0.0	0.0
Extraction-free	0	2	0	0	2	NA	100 (20-100)	33.4	0.0
Unknown									
Extracted	0	12	0	0	12	NA	100 (70-100)	0.0	0.0
Extraction-free	0	12	0	0	12	NA	100 (70-100)	0.0	0.0
Total									
Extracted	50	298	0	0	348	100 (91-100)	100 (98-100)	0.0	0.3
Extraction-free	44	274	1	1	320	97.8 (87-99)	99.6 (97-100)	3.2	5.2

% Inv, percentage of specimens reported as invalid due to internal control failure; % Pres, percentage of specimens reported as presumptive; CI, confidence interval; CT, cycle threshold; FN, false negative; FP, false positive; HDPCR, high-definition polymerase chain reaction; N, nucleocapsid; NA, not applicable; NPA, negative percent agreement; PPA, positive percent agreement; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; STM, smart transport medium; TN, true negative; TP, true positive; UTM, universal transport medium; VTM, viral transport medium.

[a]CT values obtained for extraction-free HDPCR SARS-CoV-2 N1 and N2 targets were 29.3 and 30.8, respectively. Upon repeat, only target N1 was detected with a CT value of 31.0. This specimen was reported as negative by TaqPath and HDPCR using extracted nucleic acid template.

[b]Initial and repeat test result using extraction-free HDPCR were both negative. The TaqPath result was positive and the HDPCR using extracted nucleic acid template was positive with CT values of 32.7 (N1) and 34.3 (N2).

shortages at every level of testing including swabs and transport media; plastics including pipette tips, extraction trays, and PCR plates; reagents for nucleic acid extraction; and PCR reagents and test kits.[9-11] This has resulted in the examination of alternative collection devices, transport medium, and test methods such as specimen pooling and extraction-free PCR to conserve resources and expand testing capabilities.[3,9,11-15] High test volume has also further stressed a workforce that is already understaffed, leading some laboratories to rely on research scientists to assist in laboratory testing.[9,16] We aimed to compare the analytical and clinical performance of a novel, multiplexed high-definition PCR assay (EUA HDPCR SARS CoV-2) to a widely used traditional RT-PCR assay (EUA TaqPath COVID-19) for the detection of SARS-CoV-2 in respiratory specimens. Further, we investigated the impact

of an extraction-free HDPCR protocol on analytical and clinical performance of the assay as well as the potential benefits to turnaround time, workflow, and cost.

The HDPCR assay demonstrated a slightly lower analytical LoD (ie, more sensitive) compared to TaqPath when using extracted nucleic acid template. The TaqPath assay was chosen as comparator because it is a widely adopted test with similar workflow (ie, high-complexity manual batched assay) to the HDPCR SARS-CoV-2 assay. Both assays reported 100% of test replicates as "positive" to the 10^{-2} dilution, after which specimens reported as "positive" fell to 73.3% for HDPCR and 30.0% for TaqPath (χ^2 P = .024). The increased analytical sensitivity of HDPCR could potentially translate to increased clinical sensitivity for specimens with a low viral burden, such as those collected early or late in the course

of infection.[17-20] An increase in analytical LoD (ie, less sensitive) was observed when using the extraction-free HDPCR protocol. This difference is likely multifactorial, resulting from (1) the presence of substances inhibitory to PCR within the raw specimen and (2) a 4-fold concentration of template attained during the nucleic acid extraction process. Consistent with this hypothesis, a similar dilutional effect was noted for the endogenous RP internal control target.

The clinical performance of the EUA HDPCR and TaqPath assays among 351 prospectively collected specimens tested in parallel was identical. Importantly, this encompassed 9 different transport media, suggesting no discernable impact of media type when using extracted nucleic acid template. In conjunction with the analytical LoD data, these results suggest that the novel HDPCR method is equivalent to traditional RT-PCR for detection of SARS-CoV-2.

The clinical performance of the extraction-free HDPCR protocol remained high despite the decrease in analytic LoD noted above. When compared to both TaqPath and HDPCR using extracted nucleic acid template, the extraction-free protocol demonstrated 97.8% (44/45) positive percent agreement and 99.6% (274/275) negative percent agreement among specimens with a definitive call (ie, "positive" or "negative"). The single false-positive result was reported as "negative" by TaqPath and extracted HDPCR. When repeated using the extraction-free protocol the specimen was reported as "presumptive" with only target N1 detected (CT 31.0). Based on the analytical LoD data it is unlikely that this represents a truly positive specimen that was not detected by either extracted nucleic acid test. The sole false-negative result was reported as "positive" by both TaqPath and extracted HDPCR with CT values of 32.7 (N1) and 34.3 (N2) indicating a relatively low viral load. In this instance, the decrease in LoD associated with the extraction-free protocol is consistent with a failure to accurately detect SARS-CoV-2 RNA in this specimen. Importantly, the clinical performance of any assay will be impacted by the distribution of viral loads in the population tested and the specimen type chosen, which in our study was combined nasopharyngeal/oropharyngeal swabs obtained from symptomatic or asymptomatic individuals.

Our findings are consistent with prior studies indicating that the greatest risk of false-negative result using an extraction-free protocol would be in specimens with high CT values (corresponding to low viral load), and that low viral load is more common in asymptomatic individuals.[2,3,18,19,21,22] The clinical and public health significance of patients with high CT results remains elusive. Several groups have associated high CT value with mild symptoms and/or lower risk of viral transmission,[17,19,20,22] while others have failed to demonstrate a correlation.[23] Further complicating correlation of CT value with clinical presentation is the heterogeneity of specimen collection. A specific advantage of the HDPCR SARS-CoV-2 assay over TaqPath COVID-19 is the use of the endogenous RP target as an internal control. This enables a more accurate assessment of specimen quality than the addition of exogenous MS2 template used by TaqPath; in addition to monitoring for the presence of inhibitory substances, an endogenous internal control also monitors for RNA degradation during specimen transport. Controlling for these specimen-specific factors is especially important when using an extraction-free approach. Importantly, as with any test, the positive and negative predictive value will be dependent on pretest probability; therefore, clinical symptoms and risk of exposure should be considered when interpreting individual results.

A notable drawback associated with the extraction-free protocol was a significant increase in the incidence of specimens reported as "invalid" (internal control not detected) or "presumptive" (single target, N1, or N2 detected). The overall "invalid" rate increased from 0.3% when using extracted template to 5.2% when using raw specimen. This is not unexpected because potentially inhibitory substance in the specimen and/or the transport medium are not being removed prior to PCR. Interestingly, the "invalid" rate varied significantly among the transport media tested. Approximately 28.6% (8/28) of specimens collected in STM (MedSchenker) yielded "invalid" results, which accounted for 44.4% (8/18) of all "invalid" results reported using the extraction-free protocol. Exclusion of this medium reduces the overall "invalid" rate to 2.9%, with several media (Saline, ESwab, Hardy VTM) reporting no "invalid results." The incidence of "presumptive" results also increased from 0% (0/347) when using extracted nucleic acid template to 3.3% (11/331) among all valid specimens when using the extraction-free HDPCR protocol. This translates to 19.6% (11/56) of all nonnegative (ie, "positive" plus "presumptive") results. Among specimens with a "presumptive" call, 54.5% (6/11) were "positive" by TaqPath and 45.5% (5/11) were negative, suggesting a similar rate of true and spurious single-target detection. Upon repeat testing of the 11 specimens using the extraction-free protocol, 63.6% (7/11) remained "presumptive" (3 true positive specimens, 4 true negative specimens), 1 each converted to "true" positive, 1 converted to "true" negative, 1 converted to "false"-negative, and 1 converted to "invalid." These data suggest that repeat testing of "presumptive" results with the extraction-free approach is of limited

utility, and these specimens may be better candidates for reanalysis using extracted nucleic acid template in accordance with the EUA HDPCR assay instructions for use. Of note, similar to "invalid" results, the incidence of "presumptive" result also varied among the different transport media, with 45.5% (5/11) of such results obtained from specimens collected using VTM manufactured by Genteuri. While the specific cause of the increased incidence of "invalid" and "presumptive" results associated with specific medium is not immediately apparent, these data underscore the importance of conducting a thorough evaluation of all transport media utilized by a laboratory prior to implementation of an extraction-free HDPCR approach.

Finally, an assessment of the potential benefits of an extraction-free protocol on various aspects of laboratory workflow was conducted. Use of extraction-free HDPCR resulted in a 55% reduction in hands-on time and 21% reduction in total turnaround time when compared to the standard HDPCR protocol (based on manual preparation of full 96-well extraction and PCR plates and instrument run times). Based on an average medical technologist total compensation of $45 to $55/hour, this equates to $20.25 to $24.75 savings in labor cost per plate of specimens tested. While these potential savings likely do not equate to real savings unless staffing is reduced, this labor time and associated cost can be redirected to other laboratory tasks to help reduce strain on available staff. More importantly, elimination of the manual preparation of extraction plates has the potential to reduce errors associated with repetitive pipetting and fatigue; however, our limited sample size (n = 351 specimens) did not allow us to assess for potential differences in these human error rates. Elimination of extraction also reduced consumables cost (plastics and reagents) by over $150 per 96 specimens and, more importantly, reduces the number of links in the supply chain necessary to complete the testing process. A drawback to implementation of the extraction-free protocol is the increased rate of "invalid" and "presumptive" results, which combined comprised 8.2% (29/351) of all specimens tested. These specimens would be candidates for repeat testing using a standard HDPCR protocol with nucleic acid extraction, or an alternative test platform. Of note, the "invalid" and "presumptive" rate differed by transport medium, which may provide a simple method to reduce these results by restricting the type of medium accepted for extraction-free testing.

Conclusion

The HDPCR SARS-CoV-2 assay demonstrated equivalent analytic and clinical performance to the widely used TaqPath COVID-19 assay. Use of an extraction-free HDPCR protocol demonstrated an approximately 1.0 to 2.0 log_{10} reduction in analytic LoD; however, clinical positive percent agreement and negative percent agreement of 97.8% and 99.6%, respectively, were maintained when compared to extracted TaqPath and HDPCR assays. Evaluation of the extraction-free protocol with all transport media used by the clinical laboratory is necessary to avoid increased incidence of "invalid" and "presumptive" results. Use of the extraction-free protocol results in a measurable reduction in hands on time and material cost, and reduces the potential of testing interruptions due to supply chain shortages associated with consumables required for nucleic acid extraction.

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