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Research article

Direct NP- A cost-effective extraction-free RT-qPCR based test for SARS-CoV-2

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ABSTRACT

Over 2.4 million daily total tests are currently being performed for SARS-CoV-2, in the United States. The most common SARS-CoV-2 tests require RNA extraction and purification. Extraction of RNA is a time-consuming and costly step that requires a constant supply of reagents and accessories. With the current testing demand, the supply chain remains the bottleneck for RNA extraction. Here, we report Direct NP- a cost-effective extraction-free RT-qPCR based dualplex test for SARS-CoV-2 from Nasopharyngeal (NP) swab specimens. Direct NP detects SARS-CoV-2 viral RNA from heat-denatured patient specimens using a dualplex RT-qPCR assay. Direct NP showed 92.5% positive percentage agreement (PPA) (95% Confidence Interval (CI) = 79.61%–98.43%) and 97% negative percent agreement (NPA) (95% CI = 89.11–100%) with the CDC assay. Direct NP reduces the cost per test to $2, making it suitable for broad-scale testing while lowering the cost burden on the healthcare system.

1. Introduction

Coronavirus disease 2019 (COVID-19) is caused by novel human coronavirus- severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). After the onset of the disease in Wuhan, China, in late 2019 [1], the outbreak rapidly transmitted to much of the world, and World Health Organization (WHO) declared COVID-19 a global pandemic on March 11, 2020 [2,3]. Infection with SARS-CoV-2 has resulted in over 373 million cases of COVID-19 globally with significant mortality at the time of this report [4]. In the United States, there are over 74 million confirmed cases with over 2.4 million daily tests being performed [5, 6]. Due to the rapid spread, the demand for a specific molecular diagnostic test increased globally to detect SARS-CoV-2 infection [7]. Moreover, the overlapping symptoms of COVID-19 with other acute respiratory illnesses made the diagnosis more complex and added to the already-in-demand diagnostic tests for SARS-CoV-2 [8,9]. The current methodology for the commonly used SARS-CoV-2 test involves nucleic acid extraction and subsequent detection by reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) [10]. Although it remains widely used, the nucleic acid extraction step in this assay is relatively expensive and requires technical expertise to perform the test. Challenges for broad-scale testing for healthcare providers remain twofold, 1) bottleneck in the supply chain for reagents and consumables for nucleic acid extraction, and 2) availability of qualified test personnel. Therefore, an inexpensive and simplified extraction-free test is an essential aspect for a broad-scale testing strategy that can be performed at clinical labs and flexible enough for mobile testing centers with on-site testing capability. Furthermore, there is an urgent need for a cost-effective test for detecting SARS-CoV-2 in developing countries where resources are limited with extreme surges in confirmed positive cases and worsening situations due to existing and emerging variants.

Several extraction-free methods were proposed for SARS-CoV-2 testing from various specimen types, such as NP swabs, saliva, and sputum [11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22]. These methods use a combination of enzymatic digestion with heat treatment for sample preparation before RT-qPCR. Both high demand for proteolytic enzyme and an additional step for sample processing might delay implementing broad-scale testing for laboratories. We, therefore, report Direct NP- a cost-effective and simplified extraction-free dualplex test for SARS-CoV-2 with a standalone denaturation step for nucleic acid preparation for

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RT-qPCR. We validated Direct NP on clinical NP swab specimens ($N = 73$). The modifications in Direct NP led to its estimated cost below $2/test. We validated this test on CFX96 Touch™ Deep Well Real-Time PCR Detection System (Bio-Rad) using UltraPlex 1-Step ToughMix (4X) (QuantaBio) compared to CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel [10] with modifications in instruments used for nucleic acid extraction and RT-qPCR (CDC assay).

2. Materials and methods

2.1. Sample procurement

Deidentified and blinded NP swab samples ($N = 73$) prepared in 3 ml sterile saline solution and heat-inactivated at 56 °C for 30 min were obtained from the Department of Pathology and Laboratory Medicine at Medical University of South Carolina. NP swab samples were collected and processed as described before [23]. All experiments were performed with appropriate personal protective equipment (PPE) in a biosafety cabinet.

IRB review was waived as this study is a process development and samples were deidentified and blinded.

2.2. Assay setup

A detailed assay setup with the information of reagents and consumables, primer concentrations and calculations for Direct NP is available in the **Supplementary Protocol**. Enter the number of reactions in **Supplementary reaction calculation table** to calculate reagents required for the Primer/Probe mix and the Reaction mix.

2.3. Comparator assay

Performance of Direct NP was compared to CDC assay [10] with the following modifications. Nucleic acid was extracted from 400µl NP swab samples and eluted in 50µl elution buffer using MagMAX Viral/Pathogen Nucleic Acid Isolation Kit and MAGMAX EXPRESS-96 instrument (ThermoFisher Scientific). Extracted nucleic acid samples were immediately used in the assay or stored at -80 °C for later use. RT-qPCR was performed on CFX96 Touch™ Deep Well Real-Time PCR Detection System (Bio-Rad) using TaqPath™ 1-Step Multiplex Master Mix (No ROX) (ThermoFisher Scientific).

2.4. Limit of detection (LoD)

We determined Direct NP LoD using UltraPlex 1-Step ToughMix (4X) (QuantaBio) and CFX96 Touch™ Deep Well Real-Time PCR Detection System (Bio-Rad), including denaturation step at 98 °C for 2 min. We used serially diluted SeraCare positive control spiked into a pool of 25 negative patient samples with 40, 20, 10, and 5 viral copies/µl with 20 replicates each.

2.5. Reference genomes for cross-reactivity

To rule out cross-reactivity for primer/probe combinations used in Direct NP, we obtained genomes for different strains of Human coronavirus, Betacoronavirus 1, Influenza A, Influenza B from ATCC and genomes of Bat SARS-like coronavirus, Middle East respiratory syndrome-related coronavirus, and IDT 2019-nCoV_N_Positive Control from IDT. Parameters for RT-qPCR reaction for cross-reactivity determination were identical to Direct NP.

2.6. Quantification and statistical analysis

We extracted and analyzed Cq values using the CFX Maestro software (Bio-Rad). Statistical analysis were performed in GraphPad Prism 9.0. $p \leq 0.05$ was considered significant in all statistical tests.

3. Results

3.1. Developing direct NP: a simplified and cost-effective RT-qPCR dualplex assay for SARS-CoV-2

Several US FDA EUA approved diagnostic assays, including CDC assay, require nucleic acid extraction [10, 24]. Nucleic acid extraction is a time-consuming and costly step that suffers from supply chain bottlenecks for ramping up the testing capabilities. To meet the challenges of the current testing demand for SARS-CoV-2 and avoid bottlenecks in the supply chain, we developed a simplified extraction-free dualplex RT-qPCR based assay- Direct NP. Direct NP is performed directly on the heat-denatured patient's NP specimens without the need for RNA extraction (Figure 1). Moreover, the CDC assay with three singleplex RT-qPCR reactions for each sample limits the throughput of the assay. To circumvent this issue, we designed a dualplex RT-qPCR assay that requires one reaction per sample. Both modifications, 1) extraction-free and 2) dualplex design of the Direct NP, make this assay relatively high throughput, easy to scale up, and a cost-effective alternative to diagnostic assays that involve nucleic acid extraction [24].

We used a standalone heat denaturation step to release viral nucleic acid for the RT-qPCR step to avoid supply chain backups of proteolytic enzymes used in sample preparation and for the relatively simple design. We heat-denatured samples at 98 °C for 2 min and used 5µl sample for RT-qPCR **(Supplementary Protocol)**. We found heat denaturation step alone is sufficient without losing significant sensitivity for the NP swab samples in our assay.

The CDC assay uses three primers and three probes for three targets, two for the SARS-CoV-2 nucleocapsid N1 and N2, and a Human *RNase P* (RP) in three different reactions per sample [10]. Although it remains widely used, a singleplex design limits the throughput of the assay. The sensitivity of primer/probe combinations of the CDC assay has been studied and found highly reliable in a previous study [25]. Also found in a separate study was that the results from N2 primer were not consistent when compared with N1 in a multiplex design [17]. Taking these studies into account, we designed a dualplex assay that contains two targets N1 and RP. N1 and RP primers were detected using FAM probe, and ATTO™ 647 probes respectively. These primer/probe combinations are available through IDT in the form of pre-aliquoted stocks **(Supplementary Protocol)**. It is possible to account for newly evolving variants in Direct NP assay by changing to a different primer for SARS-CoV-2 with the same FAM probe.

3.2. Analytical performance characteristics

Direct NP was evaluated for reproducibility/linear range, LoD, and specificity. To determine the reproducibility of the Direct NP over a broad range of viral copy numbers, we spiked a pool of 25 negative patient samples with serially diluted SeraCare viral control between 3-5 viral log copies/ml. SeraCare control contains a fully extractable SARS-CoV-2 genome with the viral protein coat that resembles the SARS-CoV-2 in our assay. Six serially diluted controls were tested, each with at least three technical replicates. Direct NP was reproducible with a linear response across six concentration ranges tested with an R^2-0.9793 (Figure 2). Next, we repeated the above experiment with an additional step of heat treatment at 56 °C for 30 min to determine if initial heat treatment of clinical specimens affects the reproducibility of Direct NP. We found that initial heat treatment has no significant effect on the performance of Direct NP (R^2-0.9206) (Figure 2). Based on these results, we conclude that Direct NP is highly reproducible with a linear response over a range of viral loads with or without heat treatment.

Based on the linear range, we next determined the LoD by testing the ability of Direct NP to detect 20 technical replicates using a pool of negative patient samples spiked with each of 40, 20, 10, and 5 SeraCare viral copies/µl. Direct NP was able to detect 100% of the replicates for 10 SeraCare viral copies/µl with an average N1 Cq of 37.52 (Table 1 and Figure 3). We, therefore, determined LoD for Direct NP to be 10 viral copies/µl.

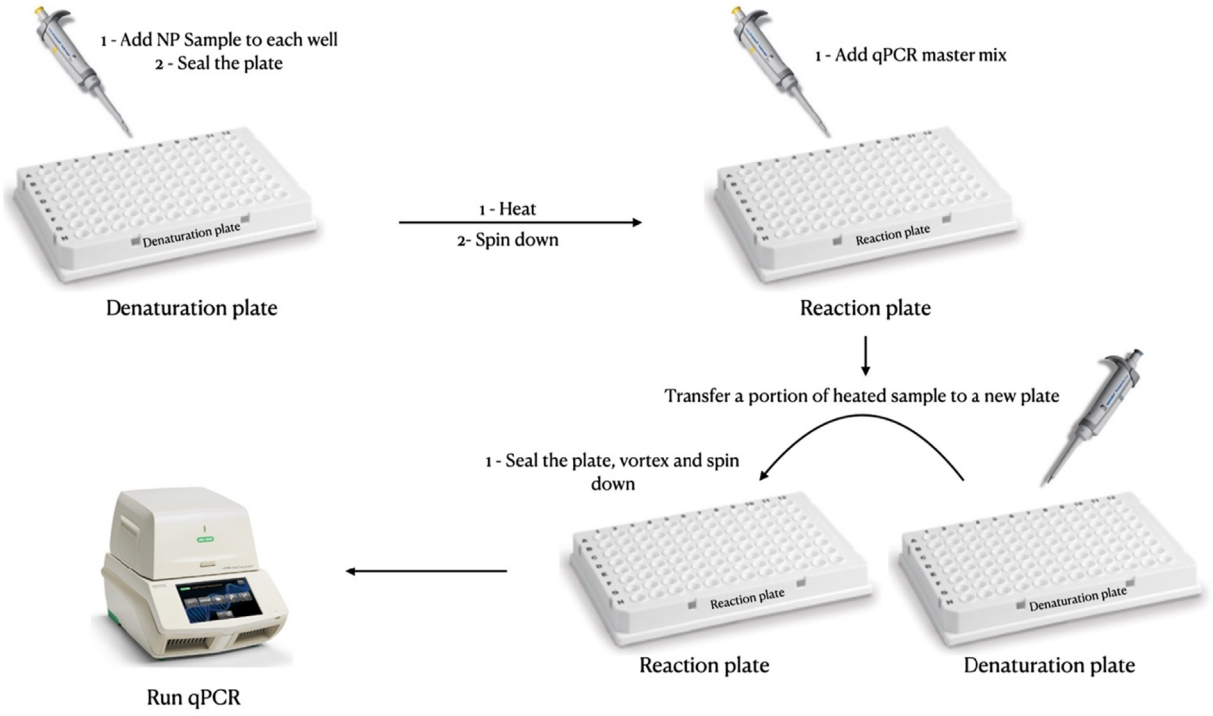

Figure 1. A schematic showing Direct NP design.

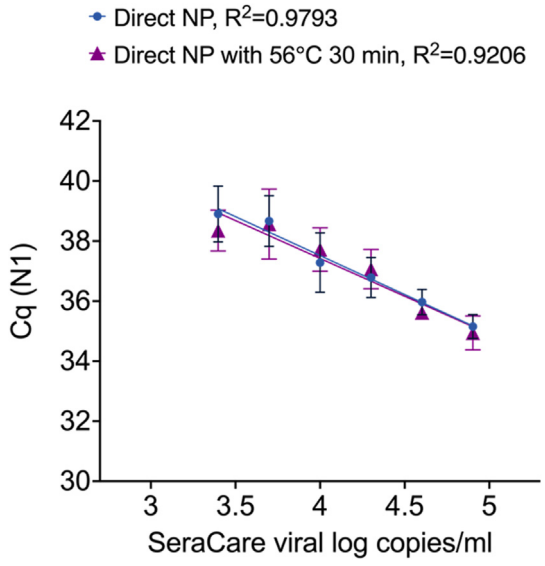

Figure 2. A semilog plot showing the linear relationship of Direct NP over a broad range of viral loads with and without heat treatment. Data points indicate an average of at least three replicates, and error bars indicate SD.

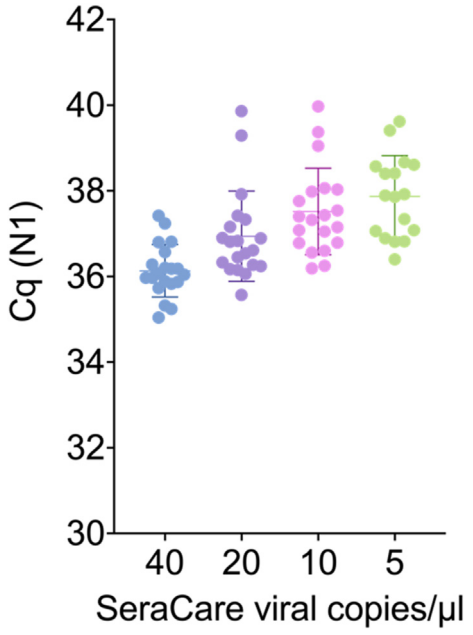

Figure 3. A scatter plot showing the mean of N1 Cq values with distribution of all values obtained for each viral copy number, and error bars indicate SD.

To confirm Direct NP does not have cross-reactivity to other closely related respiratory viruses, we tested Direct NP's N1 and RP primer/probe combination on genomes listed in Table 2. We found no false positives for any of the genomes tested, indicating the highly specific nature of N1 in an N1(FAM)-RP(ATTO™ 647) dualplex reaction. These results are consistent with CDC's findings that also showed N1 primer to be highly specific for SARS-CoV-2 with no homology with the human genome, other coronaviruses, or human microflora [10, 26].

Table 1. Lower limit of SARS-CoV-2 viral copies detection for Direct NP.

SeraCare viral copies/µl	No. of replicates detected/No. of replicates tested (%)	Average Cq	SD
40	20/20 (100)	36.13	0.61
20	20/20 (100)	36.94	1.05
10	20/20 (100)	37.52	1.01
5	17/20 (85)	37.87	0.95

3.3. Clinical validation

Direct NP was validated using the CDC qPCR assay with 73 matched NP swab specimens. RNA extraction was performed using MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit for the singleplex CDC assay.

Table 2. Cross-reactivity for Direct NP. For each viral genome, we tested three technical replicate.

Virus	Strain	Catalog#	2019-nCoV_N1	Result
Human coronavirus	229E	ATCC#VR-740DQ	0/3	Negative
Human coronavirus	NL63	ATCC#VR-3263SD	0/3	Negative
Human coronavirus	HKU1	ATCC#VR-3262SD	0/3	Negative
Betacoronavirus 1	OC43	ATCC#VR-1558DQ	0/3	Negative
Influenza A	A/Virginia/ATCC1/2009	ATCC#VR-1736DQ	0/3	Negative
Influenza B	B/Wisconsin/1/2010 BX-41A	ATCC#VR-1885DQ	0/3	Negative
Bat SARS-like coronavirus	bat-SL-CoVZC45	IDT#10006624	0/3	Negative
Middle East respiratory syndrome-related coronavirus	KNIH/002_05_2015	IDT#10006623	0/3	Negative
IDT 2019-nCoV_N_-Positive Control	SARS-CoV-2 isolate Wuhan-Hu-1	IDT#10006625	3/3	Positive

The extracted RNA was used for RT-qPCR reaction using TaqPath™ 1-Step Multiplex Master Mix (No ROX) (ThermoFisher Scientific) and CFX96 Touch™ Deep Well Real-Time PCR Detection System (Bio-Rad). For CDC singleplex assay, we used the reaction mixture and instrument settings specific for TaqPath™ 1-Step Multiplex Master Mix (No ROX) as reported in the CDC assay [10]. The median N1 Cq value for Direct NP was 2.06 higher (P-value <0.0001, two-tailed t-test) than the median N1 Cq value for CDC assay. Direct NP had higher median Cq value for N1 because CDC assay used extracted RNA that is eight times more concentrated than Direct NP samples. Direct NP had 92.5% PPA (95% CI = 79.61%–98.43%) and 97% NPA (95% CI = 89.11–100%) when compared with the CDC assay (Table 3). Out of three positive samples in CDC assay that Direct NP did not detect, two samples had high N1 Cq values 39.26 and 36.11, and one sample had N1 Cq value 33.50 in CDC assay (Figure 4). The possible reasons for no detection of N1 in Direct NP for the sample with N1 Cq value 33.50 in CDC assay could be PCR inhibitors or sample degradation. One sample was found negative for both N1 and RP and hence considered invalid.

3.4. Result interpretation

Figure 5 shows result interpretation tree for Direct NP. Similar Direct NP result interpretation is also shown in Table 4.

3.5. Cost calculation

Direct NP is a scalable, relatively high throughput, and cost-effective assay for SARS-CoV-2. The test maintains significant assay sensitivity while saving time and avoiding supply chain bottlenecks for expensive reagents and consumables for nucleic acid extraction. As a result, the estimated cost per reaction of Direct NP is less than $2. Table 5 shows the breakdown of each Direct NP assay component's price and the final cost per reaction. This price includes reagents and consumables excluding instrument, labor, and other miscellaneous expenses.

Table 3. Direct NP assay agreement compared to modified CDC assay in matched NP swab specimens. One sample was deemed invalid and was not considered for agreement calculations.

		CDC assay	
		Positive	Negative
Direct NP	Positive	37	0
	Negative	3	32
Total		40	33
PPA = 92.5% (37/40)	NPA = 97% (32/33)		

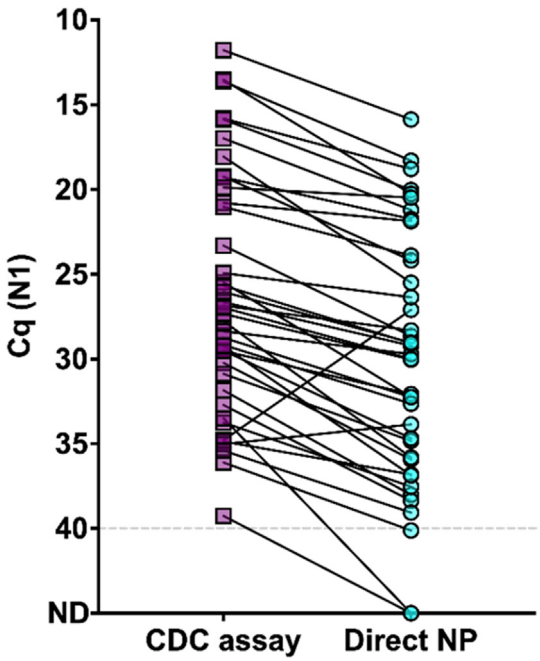

Figure 4. Direct NP N1 Cq values compared with modified CDC assay in matched NP swab specimens. Cq value for sample with undetected virus was set at 45.

4. Discussion

Current CDC mitigation strategies for communities with local SARS-CoV-2 transmission are based on promoting behavior and effectively communicating safe practices, including time-based isolation for people with symptoms that prevent spread until broad-scale testing is implemented [27, 28]. Detecting the virus in broad-scale testing could help stop its community transmission and allow the reopening of businesses and schools. However, as the demand for SARS-CoV-2 testing increases, managing supplies and cost of RNA extraction reagents and consumables becomes increasingly challenging for healthcare providers. This cost burden directly limits the scale and throughput of the diagnostic test and remains one of the main constraints for implementing broad-scale testing. Direct NP is a cost-effective RNA extraction-free RT-qPCR based test for SARS-CoV-2 performed directly on heat-denatured NP swab specimens.

Several methods used combinations of treatments for releasing viral RNA into the reaction for various types of samples. For example, either enzymatic or combination of enzymatic and heat denaturation have been used for Saliva, Sputum, and NP swab samples [11, 12, 13, 15, 16, 17, 29]. Our data showed that heat denaturation for 2 min alone is sufficient for detecting viral RNA into the sample with no significant loss of PPA when compared to CDC assay (Table 3). This modification allowed us to simplify the test without the delays of enzyme supplies due to high demand. The extraction-free test was one of the two modifications of our assay. We also designed a dualplex assay, a second modification. We used CDC primers for two targets virus nucleocapsid (N) gene (N1) for virus detection and human RNase P gene as control with two different fluorescent probes FAM and ATTO™ 647, respectively. We validated Direct NP by comparing it with singleplex CDC assay with extracted RNA on the same samples. Dualplex design allows Direct NP to be a single tube test.

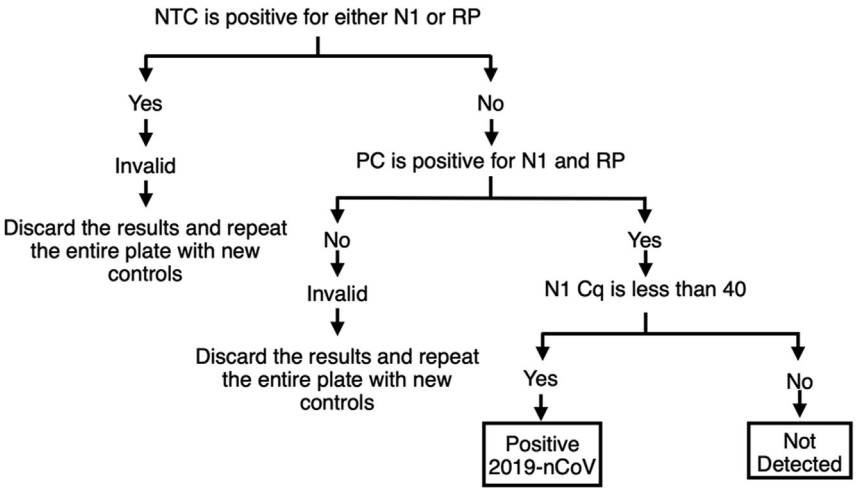

Figure 5. Direct NP result interpretation tree.

Table 4. Direct NP interpretation of results.

2019 nCoV_N1	RP	Result interpretation	Report
+	±	2019-nCoV detected	Positive 2019-nCoV
—	+	2019-nCoV not detected	Not Detected
—	—	Invalid	Invalid (Action: Repeat the test. If the repeat test is still negative for N1 and RP, collect a new specimen)

Table 5. Direct NP cost estimation.

Item	Cost
Primers and Probes	$21.44
QuantaBio UltraPlex 1-Step ToughMix (4X) (QuantaBio) Master Mix	$130.37
SeraCare Control	$0.32
Consumables	$28.00
Total/96 reactions	$180.13
Per reaction (20μl) cost	$1.88

This modification allowed higher throughput of the assay when compared to singleplex CDC assay. With both changes, we were able to keep the test's cost relatively low to nearly $2/test.

The validation data in our test, including denaturation at 98 °C for 2 min, is master mix-instrument specific. We used CFX96 Touch™ Deep Well Real-Time PCR Detection System (Bio-Rad) and UltraPlex 1-Step ToughMix (4X) (QuantaBio). We strongly recommend performing this test using this combination of master mix-instrument. For a different master mix or instrument, a new validation is needed. Direct NP also offers the flexibility of changing the SARS-CoV-2 target for accounting newly emerging variants. Furthermore, as travel, schools, and other businesses start to opening up, we predict an increase in demand for testing and repeat testing. To this end, Direct NP reduces the cost burden on the healthcare system due to its low cost of less than $2/test.

5. Conclusion

We developed a scalable, and cost-effective extraction-free dualplex RT-qPCR-based test for SARS-CoV-2. This test is performed directly on denatured NP swab samples and does not require expensive kits and reagents for RNA extraction. This simplified, inexpensive, and yet sensitive test will help healthcare providers meet current and future testing demand and help implement broad-scale testing to detect and stop the virus transmission.

5.1. Limitations of the study

In the present study, we showed a standalone denaturation step in an extraction-free, dualplex SARS-CoV-2 test is sufficient for releasing SARS-CoV-2 viral RNA into an RT-qPCR reaction with 92.5% PPA compared to CDC assay. However, the clinical specimens were deidentified and blinded and previously denatured at 56 °C for 30 min for safe transport to the testing laboratory. The scope of further improving PPA in our test remains to be seen if performed on specimens that are not denatured before their transport to the testing laboratory.

Declarations

Author contribution statement

Rasesh Y. Parikh: Conceived and designed the experiments; performed the experiments; analyzed and interpreted the data; wrote the paper.

Vamsi K. Gangaraju: Conceived and designed the experiments; analyzed and interpreted the data; wrote the paper.

Shikhar Mehrotra: Conceived and designed the experiments; contributed reagents and materials; wrote the paper.

Satish N. Nadig and Philip H. Howe: Conceived and designed the experiments; wrote the paper.

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Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

Supplementary content related to this article has been published online at https://doi.org/10.1016/j.heliyon.2022.e09735.

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