Detection of SARS-CoV-2 RNA by direct RT-qPCR on nasopharyngeal specimens without extraction of viral RNA

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Abstract

To circumvent the limited availability of RNA extraction reagents, we developed a protocol for direct RT-qPCR to detect SARS-CoV-2 in nasopharyngeal swabs. Incubation of specimens at 65°C for 10 minutes along with the use of TaqPath™ 1-Step RT-qPCR Master Mix provides higher analytical sensitivity for detection of SARS-CoV-2 RNA than many other commercial and laboratory-developed methods. In 132 specimens submitted for SARS-CoV-2 testing, the sensitivity, specificity and accuracy of our optimized approach were 95%, 99% and 98.5%, respectively, with reference to results obtained by a standard approach involving RNA extraction. Also, the RT-qPCR Cₗ values obtained by the two methods were highly correlated.
The ongoing pandemic of coronavirus disease (COVID-19) caused by a novel coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has led to a worldwide shortage of diagnostic test kits and reagents. Since the virus was identified and its genome sequenced in early January 2020, detection of viral RNA in respiratory specimens by real-time reverse transcription PCR (RT-qPCR) remains the main approach to manage the outbreak by allowing early detection of cases and targeted measures to prevent transmission of the virus [1, 2]. The massive demand for SARS-CoV-2 RT-qPCR has brought about a global shortage and supply chain irregularities of RNA extraction kits that are crucial for RT-qPCR testing [3, 4]. In this study, we tested a number of commercial and laboratory-developed, specimen pre-treatment procedures to optimize the performance of direct RT-qPCR for SARS-CoV-2 avoiding the RNA extraction step. This method was validated against a standard approach that included extraction of viral RNA on a commercial automated extraction platform.

### Optimizing specimen pre-treatment methods for direct RT-qPCR for SARS-CoV-2

Using a human coronavirus HKU1 (hCoV-HKU1) positive specimen as a surrogate for SARS-CoV-2, we first assessed whether specimens can be used directly for RT-qPCR after 2-10-fold dilution with nuclease free water (NFW), simple heat treatment (100°C for 5 min) and centrifugation to remove any insoluble material that may be present in the specimen. The pre-treated specimens were then assessed in parallel with extracted specimens by a previously described, laboratory developed RT-qPCR for hCoV-HKU1 (Table S1). A significant loss of sensitivity was observed with a RT-qPCR ΔC_T ranging from 10-14 (Table S2). To determine whether any components of universal transport medium (UTM) (Copan Diagnostics) were inhibitory to RT-qPCR, we collected nasopharyngeal (NPFS) from laboratory volunteers in NFW along with swabs in UTM. We then spiked a SARS-CoV-2 positive specimen to all specimens and assessed them by SARS-CoV-2 RT-qPCR. However, no significant improvement in sensitivity was observed (Table S3).

Similar results were observed when two commercial test kits were used for direct RT-qPCR: Arcis Coronavirus RNA extraction research kit comes with lysis reagents that can be used directly in RT-qPCR; and Takara PrimeDirect™ Probe RT-qPCR kit provides a master mix that is compatible with heat-treated specimen extracts. However, in our evaluation both test kits failed to demonstrate an acceptable level of sensitivity (Tables S4 and S5). Our attempts to further optimize the pre-treatment conditions showed modest improvement with a non-ionic detergent, Tween-20, and further improvement with a heating step at 65°C for 10 min without centrifugation (ΔC_T = 5.2) (Table S6). We then tested this low heat approach with more SARS-CoV-2 positive specimens and using 3 different RT-qPCR master mixes. Interestingly, we found that with TaqPath™ 1-Step RT-qPCR Master Mix, 4/4 positive samples were correctly detected with a ΔC_T range 0.8 – 3.8 (Table 1). On the other hand, two other master mixes gave higher ΔC_T and 1/4 positive results were missed by both. Based on these results, the optimal pre-treatment and reaction conditions for the direct approach were: i) transfer and dilute (4-fold) 10 µl of NPFS specimen in NFW; ii) incubate at 65°C for 5 min; and iii) test 8 µl of heat lysed specimen in a 20 µl reaction using TaqPath™ 1-Step RT-qPCR Master Mix.

### Clinical validation of optimized, direct RT-qPCR approach

A total of 132 NPFS specimens that were previously tested with standard approach including viral RNA extraction were re-tested with the new direct approach. The direct approach detected all except one positive case with C_T >38. On the other hand, the direct approach detected (C_T >37) SARS-CoV-2 in one specimen that was negative by standard approach. Overall agreement of results between two approaches was >98%. The sensitivity and specificity of the new approach compared to the reference method were 95% and 99%, respectively (Table 2). The RT-qPCR C_T values for all specimens that were positive by both methods (n=18) were moderately correlated with a Pearson coefficient (R) of 0.6971 (p<0.01) (Figure 1). The rate of PCR inhibition among the specimens that gave negative RT-qPCR results by the direct approach was 8% compared to 9% by the standard approach.
Discussion and conclusions

Since the emergence of the virus in late 2019, SARS-CoV-2 has infected more than 2 million people and caused the death of more than 150,000 people worldwide. The WHO and many other health authorities around the world have emphasized the critical role of laboratory testing in case management, surveillance, and rapid response, and infection prevention and control [5, 6]. As such, hundreds of thousands of RT-qPCR tests are being performed daily across the globe, leading to a shortage of RNA extraction reagents and RT-qPCR kits [7, 8]. Here, we present a simple solution for direct RT-qPCR on nasopharyngeal specimens for laboratories facing a shortage of RNA extraction reagents.

Success in RT-qPCR testing depends on multiple factors. RNA extraction is preferable to the use of direct specimens because the extraction process concentrates and purifies the RNA targets and excludes PCR inhibitory substances. The use of pre-treated or untreated specimens directly in RT-qPCR is challenging because of the presence of inhibitors and RNA loss due to heating and/or RNases. After many attempts with various pre-treatment agents and conditions, we have determined an optimal pre-treatment protocol complemented with specific RT-qPCR reagents, that generates results equivalent to standard methods that involve RNA extraction. Minimizing RNA loss through the low heat approach, appropriate dilution of inhibitory substances and the higher sensitivity of TaqPath™ 1-Step RT-qPCR Master Mix may have played a combinatorial role in achieving equivalency of the direct RT-qPCR compared to a standard approach requiring viral RNA extraction.

In summary, our new approach demonstrated high sensitivity and specificity in the detection of SARS-CoV-2 RNA, and the rate of RT-qPCR inhibition was similar to that of a standard approach. By skipping the RNA extraction step, the new approach will also significantly reduce the cost and improve the turn-around time of the assay.
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Figure 1: Correlation of RT-qPCR C\textsubscript{T} values obtained by optimized versus standard approach
Table 1: Direct RT-qPCR on SARS-CoV-2 positive and negative NPF S specimens after heating at 65°C for 5 minutes with different RT-qPCR master mixes

| Sample No. | SARS-CoV-2 Ct |
|------------|---------------|
|            | Standard method | Quantifast Pathogen RT-PCR + IC Master Mix | PrimeDirect™ Probe RT-qPCR Mix | TaqPath™ 1-Step RT-qPCR Master Mix |
| 1          | 21.5           | 29.4           | 24.6           | 22.8           |
| 2          | 34.5           | Undetermined   | Undetermined   | 35.3           |
| 3          | 24.5           | 30.4           | 28.7           | 25.5           |
| 4          | 22             | 29.9           | 31.4           | 25.8           |
| 5          | Undetermined   | Undetermined   | Undetermined   | Undetermined   |

NPF S specimens were either subjected to viral RNA extraction by standard method using the NuclISENS easyMAG automated extraction system (bioMerieux), or diluted 4-fold with NFW followed by incubation at 65°C for 5 minutes. All samples were tested for SARS-CoV-2 RNA by standard RT-qPCR using different master mixes in duplicate and mean Ct values were compared.

Table 2: Performance of optimized direct RT-qPCR approach with reference to standard approach for detection of SARS-CoV-2 RNA

| Statistic            | Value | 95% CI     |
|----------------------|-------|------------|
| Total number of specimens | 132   | -          |
| True positive        | 18    | -          |
| True negative        | 112   | -          |
| False positive       | 1     | -          |
| False negative       | 1     | -          |
| Sensitivity          | 95.0% | 74% to 99.8% |
| Specificity          | 99.0% | 95.2% to 99.9% |
| Accuracy             | 98.5% | 94.6% to 99.8% |