Extraction-free SARS-CoV-2 detection by rapid RT-qPCR universal for all primary respiratory materials

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ABSTRACT

Background: Fast and reliable detection of SARS-CoV-2 is crucial for efficient control of the COVID-19 pandemic. Due to the high demand for SARS-CoV-2 testing there is a worldwide shortage of RNA extraction reagents. Therefore, extraction-free RT-qPCR protocols are urgently needed.

Objectives: To establish a rapid RT-qPCR protocol for the detection of SARS-CoV-2 without the need of RNA extraction suitable for all respiratory materials.

Material and methods: Different SARS-CoV-2 positive respiratory materials from our routine laboratory were used as crude material after heat inactivation in direct RT-qPCR with the PrimeDirect™ Probe RT-qPCR Mix (TaKaRa). SARS-CoV-2 was detected using novel primers targeted to the E-gene.

Results: The protocol for the detection of SARS-CoV-2 in crude material used a prepared frozen-PCR mix with optimized primers and 5 μl of fresh, undiluted and pre-analytically heat inactivated respiratory material. For validation, 91 respiratory samples were analyzed in direct comparison to classical RNA-based RT-qPCR. Overall 81.3 % of the samples were detected in both assays with a strong correlation between both Ct values (r = 0.8492, p < 0.0001). The SARS-CoV-2 detection rate by direct RT-qPCR was 95.8 % for Ct values < 35. All negative samples were characterized by low viral loads (Ct > 35) and/or long storage times before sample processing.

Conclusion: Direct RT-qPCR is a suitable alternative to classical RNA RT-qPCR, provided that only fresh samples (storage < 1 week) are used. RNA extraction should be considered if samples have longer storage times or if PCR inhibition is observed. In summary, this protocol is fast, inexpensive and suitable for all respiratory materials.

1. Introduction

The pandemic spread of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) causing the novel coronavirus disease 2019 (COVID-19) is a global challenge.

Early identification and sequencing of the virus enabled a fast development of protocols for the detection of viral RNA in respiratory specimens by real-time reverse transcription PCR (RT-qPCR) to manage the outbreak by allowing early detection of cases and taking appropriate measures to prevent the transmission of the virus [1,2]. The massive demand for SARS-CoV-2 RT-qPCR testing, also recommended by the WHO resulted in a worldwide shortage of diagnostic reagents including RNA extraction kits which is still a major challenge [3].

To overcome the lack of reagents and to increase the capacities for SARS-CoV-2 testing various approaches like pooling strategies, direct RT-qPCR using primary material or isothermal methods are currently being established [4–8]. The biggest challenge is to maintain analytical sensitivity and to deal with different respiratory specimens. The aim of this study was to establish a rapid protocol of a direct RT-qPCR for the detection of SARS-CoV-2 without the need for prior RNA extraction. In addition, this method should be independent of the respiratory material used while maintaining detection sensitivity.

2. Material and methods

2.1. Respiratory specimens

All specimens used to establish the direct RT-qPCR for the detection of SARS-CoV-2 were part of routine diagnostic detected to be positive for SARS-CoV-2 by RNA-based RT-qPCR with the protocol by Corman and colleagues [1] or by the cobas® SARS-CoV-2 test on the cobas® 6800 sytem (Roche). A total of 36 different respiratory samples were

used to establish the protocol for the detection of SARS-CoV-2 in crude specimen. The validation of the protocol was performed with 91 different respiratory samples, including nasopharyngeal or throat swabs (n = 78), tracheal secretion (TS = 8), bronchoalveolar lavage fluid samples (BAL = 2), aspirate (AS = 2) and saliva (S = 1) (Table S1). Viscous samples were pre-incubated with Remel™ Sputasol (Thermo Scientific™).

2.2. Pre-analysis

For a direct comparison of direct RT-qPCR to the gold standard RT-qPCR using purified nucleic acid, total nucleic acid was extracted by the EZ1 platform with the EZ1 Virus Mini Kit v2.0 (Qiagen) using 200 µl sample volume eluted in 60 µl buffer. The input of purified nucleic acid for the RT-qPCR was adapted equivalent to the source material with a dilution factor of 3.33 using nuclease-free water. For the analysis of crude specimen, 50 µl of respiratory material was heat inactivated at 99 ˚C for 5 min and centrifuged at 4000 rpm for 5 min to ensure safe working and lysing of virus and cells.

2.3. SARS-CoV-2 RT-qPCR

RT-qPCR was performed using the PrimeDirect™ Probe RT-qPCR Mix (TaKaRa), which is designed for one-step real-time RT-qPCR without the need of a pre-analytic extraction step. To reduce hands on time PCR mixes were prepared in large batches and frozen ("frozen-PCR mixes"). Per reaction 25 µl RT-qPCR mixture containing 1x PrimeDirect™ RT-qPCR, 200 nM probe and 400 nM each primer (Table 1) were aliquoted in 8-strips and stored at −20 ˚C until usage. For amplification, tubes were thawed and 5 µl heat-inactivated respiratory material were used for RT-qPCR. Reverse transcription condition were 30 s at 95 ˚C and 5 min reverse transcription at 60 ˚C followed by 45 qPCR cycles each 5 s 95 ˚C and 30 s annealing/extension at 60 ˚C. RT-qPCR cycling was performed on a QuantStudio 5 Real-Time PCR System (Applied Biosystems). Samples were considered positive when a signal was detected, negative if only the internal control was amplified and invalid when internal control was negative.

2.4. Statistical analyses

Statistical analyses were performed using GraphPad Prism 8.0.2. For the comparison of matching samples a paired *t*-test was performed and correlations were calculated using Pearson correlation.

3. Results

The PrimeDirect™ Probe RT-qPCR SARS-CoV-2 protocol has different requirements for each respiratory material and therefore is unsuitable for routine diagnostics of clinical care. Thus, we first optimized the protocol with the aim to be suitable for all respiratory materials including swabs in different transport media, tracheal secretions, aspirates, saliva and bronchoalveolar lavages.

In a first step, we reduced the size of the PCR product by generating new primers and probe optimized to the short thermal protocol recommended for the PrimeDirect™ kit. In our SARS-CoV-2 routine diagnostics we use the recommended Sarbeco primer-probe set located in the E-gene of SARS-CoV-2 with a product size of 113 bp [1]. The newly designed primers and probe are also located in the E-gene with overlaps to the Sarbeco set but with a product size of only 103 bp (Table 1).

3.1. Validation of PCR conditions

After validation of primers and probes the efficiency of the new E-gene CoV-2 primers were compared to the Sarbeco E-gene primers. To do so, we first compared the detection of viral RNA in 20 respiratory samples from confirmed SARS-Cov-2 positive patients both, by Sarbeco E-gene and our CoV-2 E-gene primers using the PrimeDirect™ RT-qPCR kit. (Fig. 1a). The new developed CoV E-gene primers lead to a significant increase in sensitivity with a mean decrease of ΔCt 1.0 (ΔCt range 0.2–3.7; p = 0.0005; Fig. 1). Additionally, two samples (one swab and one sputum) with Ct values > 35 were exclusively detected with the new CoV E-gene primers.

To further establish a streamlined workflow, we also explored preparation and storage of large "ready-to use" PCR-mixes ("frozen-PCR-mixes"). Comparison of freshly prepared mixes to the frozen PCR-mixes which were stored at −20 ˚C for 1 week showed no significant differences in Ct values (Fig. 1b), which confirms the efficiency of frozen PCR-mixes for the detection of SARS-CoV-2.

3.2. Crude specimen optimization

RNA extraction is expensive, time-consuming and one of the most-limited reagents in the current pandemic. Several reports indicate that RT-qPCR on crude specimen without purification is feasible, however needs optimization [5,9,10].

To do so, the influence of source material, sample storage, sample dilution, sample input volume and time of heat inactivation on analytical sensitivity was analyzed (Fig. 2). The performance of each optimisation step was evaluated by comparison to classical RNA-based RT-qPCR and given as ΔCt.

In order to determine the optimal duration of the heat inactivation step, that is sufficient for cell and virus lysis while not degrading RNA, three patient samples with Ct values 16.1 (TS), 23.4 (swab) and 26 (TS) were heat treated for 1, 2, 5 and 10 min at 99 ˚C. Heat inactivation for 1 and 2 min seemed sufficient for tracheal secretions however not for the swap sample with ΔCt 3 and 1.2 compared to extracted RNA, respectively. Heating to 99 ˚C for 5 and 10 min resulted in a more uniform detection pattern with ΔCt 0.7 and 0.9 (Fig. 2). Since ΔCt between 5 and 10 min did not differ significantly, all further inactivations we carried out for 5 min to save time.

Next, we analyzed if an increase in crude sample material input increases detection of SARS-CoV-2. Increasing the sample input gradually lowered the Ct values with ΔCt up to 2.0. However, to keep the risk of potential PCR inhibition to a minimum 5 µl was used in all further validation steps. To analyze if potentially inhibitory factors can be reduced, 1:2, 1:4 and 1:5 dilutions of the primary samples in

Table 1
Primers and probes for the detection of SARS-CoV-2 and an internal control by direct RT-qPCR.

Name	Target	Sequence*	Concentration/reaction
CoV-E-F	E gene	CTTTTTCTTGCTTTCGTGGTATTCT	400 nM
CoV-E-R	E gene	TACAAGACTCACGTTAACAATATTGCA	400 nM
CoV-E-Pr	E gene	FAM-CTAGCCATCCTTACTGCGCTTCGATTGTG-BHQ	200 nM
HBV-Taq1	HBV-SynQ	CAACCTCCAATCACTCACCAAC	200 nM
HBV-Taq2	HBV-SynQ	ATATGATAAAACGC GCAGACAC	200 nM
HBV-IC	HBV-SynQ	Cy5-CTGCCGAGCTCTGACTA-BHQ	200 nM

HBV-SynQ (internal control): a synthetical plasmid coding for an inactivated s-Antigen of Hepatitis B.

* Reference strain: Severe acute respiratory syndrome coronavirTs 2 isolate WThan-HT-1, complete genome. Accession MN908947.
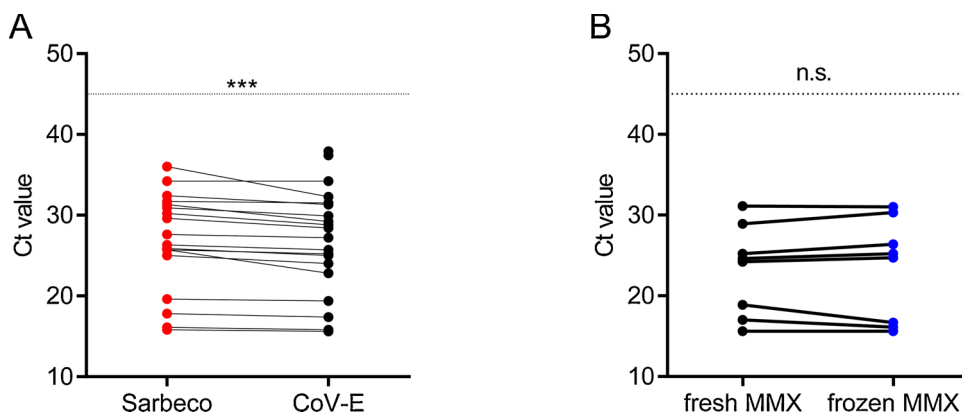
Fig. 1. Analysis of PCR conditions for the detection of SARS-CoV-2 by direct RT-qPCR.

(A) Direct RT-qPCR with the Sarbeco and the new CoV-E primers.

Patient matched respiratory crude samples (n = 20) including swabs, tracheal secretion, brochoalveolare lavage and sputum were analyzed for the detection of SARS-CoV-2 by direct RT-qPCR with the Sarbeco primers and the new CoV-E primers.

(B) Direct RT-qPCR with fresh and pre-frozen PCR-mixes.

Patient matched respiratory crude samples (n = 8) including swabs, tracheal secretion and BAL were analyzed for the detection of SARS-CoV-2 by direct RT-qPCR with either fresh prepared master-mixes (MMX) or pre-frozen master-mixes.

Dotted line: detection limit by performed qPCR cycles; *** (p ≤ 0.001); n.s. (not significant).

nuclease free water were analyzed (Fig. 2). The dilutions showed no reduction of inhibitory factors, as the mean ΔCt increased by 1.1, 0.9 and 1.3, respectively. Thus, undiluted respiratory material was the material of first choice.

Finally, the influence of sample storage conditions was analyzed. Therefore, crude specimens were stored overnight at 4 °C or were frozen at -20 °C and subsequently used in direct RT-qPCR. As shown in Fig. 2 storage at -20 °C resulted in a mean ΔCt of 15.7 compared to ΔCt 0.4 for fresh specimens stored at 4 °C not longer than overnight. Thus, storage of crude specimen at -20 °C is not recommended.

Since we observed this drastic increase in ΔCt when samples were frozen before direct RT-qPCR, the stability of samples over a period of 4 weeks at 4 °C was analyzed. As illustrated long-term storage at 4 °C significantly increased the ΔCt value in direct RT-qPCR (Fig. 3). In detail, 26/32 of the analyzed samples (81.3 %) have shown an increase of ΔCt > 3 after long-term storage at 4 °C with a mean ΔCt increase of 8.6 (ΔCt range 3.1–14.5). Of note, a total of 11 samples could no longer be detected after long-term storage.

In summary, the optimal performance is achieved with 5 μl fresh sample and heat-inactivated for 5 min at 99 °C. For all following experiments this protocol was applied.

3.3. Validation of the direct RT-qPCR with respiratory samples

After optimization the sensitivity of the direct RT-qPCR protocol was verified with 91 respiratory samples which were detected positive for SARS-CoV-2 either by in-house RT-qPCR with the protocol by Corman and colleagues [1] or by using the cobas® SARS-CoV-2 test (Roche). For direct comparison 1.5 μl extracted RNA was used as input volume which is equivalent to 5 μl input volume of crude specimen. Overall 74/91 samples (81.3 %) were detected in both, extracted RNA as well as crude material with a significant correlation of Ct values (r = 0.8492, p < 0.0001; Fig. 4). Interestingly, one sample identified as negative in the RNA RT-qPCR, was detected by direct RT-qPCR with a Ct value of 34.1. Of the remaining samples, 16 (17.6 %) were positive in RNA RT-qPCR however, not in the direct RT-qPCR (Table S1). In detail, 10/16 direct-negative samples had Ct values > 35 (Ct value range 35.4–40.2), while 5/16 negative samples had Ct values < 35 (Ct value range 24.4–34.9; Table S1). Only 1 out of 91 samples (1.1 %) was negative for SARS-CoV-2 and negative for the internal control and therefore invalid (Table S1). Of note, all undetected samples had in common that they were swab samples and were stored for 2–6 weeks at 4 °C before direct RT-qPCR was performed. No detection failures were observed with other respiratory materials.

After successful validation the protocol was implemented in our routine diagnostic for staff surveillance. Notably, between 6th June and 11th July 2020, in total 523 samples have been screened using the Prime Direct protocol and only 0.6 % of the samples were invalid which demonstrates the good implementation capability and reliability of this method.

4. Discussion

Direct RT-qPCR for the detection of SARS-CoV-2 without the need for prior RNA extraction is one possibility to overcome the shortage of extraction reagents. The PrimeDirect™ Probe RT-qPCR Mix used in this study is suitable for all respiratory materials, had a similar detection rate compared to extracted RNA and is very fast (47 min cycling). Several reports indicate that RT-qPCR are compatible with direct testing of nasopharyngeal and oropharyngeal swab specimens without a prior purification or extraction step, but these studies only analyzed swab samples or had insufficient sensitivity [5,9–12].

Based on our optimization, the following conditions are suitable for all respiratory materials from our routine: (1) primers and probes optimized for short cycling conditions; (2) heat inactivation of primary material at 99 °C for 5 min; (3) input volume of 5 μl fresh and undiluted respiratory material.

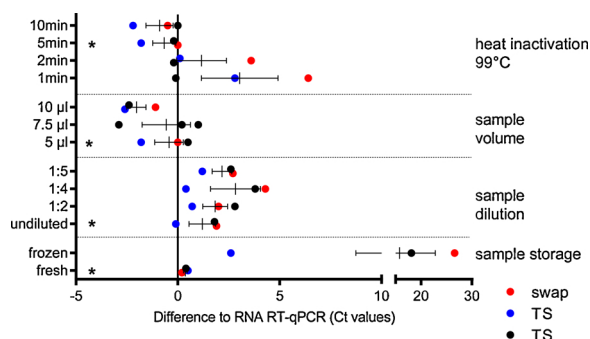Importantly, we could confirm that storage of the respiratory

Fig. 2. Analysis of different sample conditions for optimization of the direct RT-qPCR protocol for the detection of SARS-CoV-2 in respiratory crude samples. For the direct comparison of different sample conditions three patients samples with RNA Ct values 16.1 (TS, blue), 23.4 (swab, red) and 26 (TS, black) were selected. Ct values are shown in difference to RNA RT-qPCR Ct values (ΔCt). Mean Ct values with SEM are indicated.

TS: tracheal secretion; frozen: storage at -20 °C before sample processing; fresh: storage at 4 °C < 24 h; *: selected condition for direct RT-qPCR protocol; SEM: standard error of mean.
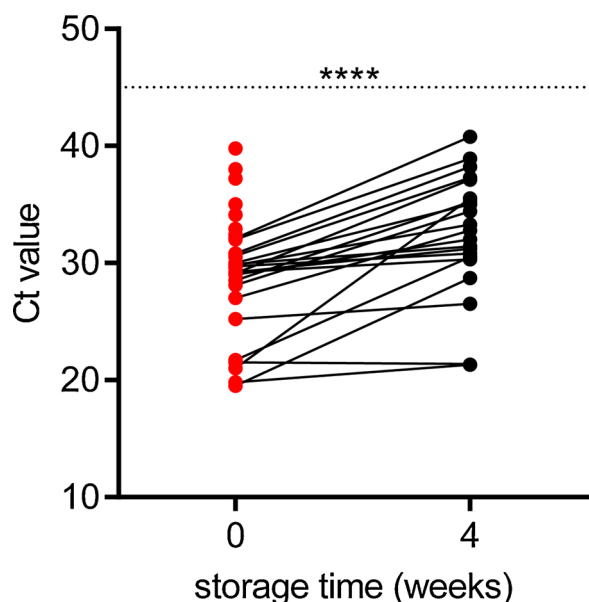
Fig. 3. Impact of long-term storage of respiratory samples at 4 °C on SARS-CoV-2 detection by direct RT-qPCR.
Patient matched respiratory samples (n = 32) including swabs, tracheal secretion, bronchoalveolare lavage and saliva were analyzed for the detection of SARS-CoV-2 by direct RT-qPCR at the timepoint of sample receipt (week 0) and 4 weeks after storage at 4 °C (week 4).
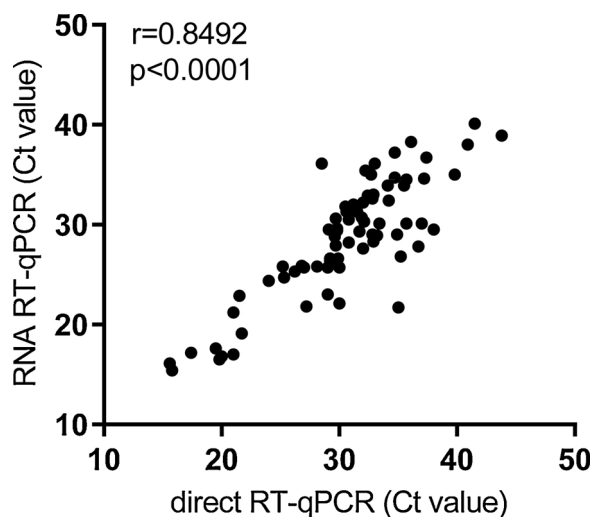Dotted line: detection limit performed qPCR cycles; ****($p \leq 0.0001$).

Fig. 4. Correlation of Ct values obtained by direct RT-qPCR versus classical RT-qPCR with extracted RNA for the detection of SARS-CoV-2.
Respiratory samples (n = 91) including swabs, tracheal secretion, bronchoalveolare lavage, aspirate and saliva were analyzed for the detection of SARS-CoV-2 by RT-qPCR with crude specimen and RNA-based RT-qPCR. Ct values were correlated.

samples at -20 °C before RT-qPCR significantly reduces sensitivity, as already has shown by Merindol and colleagues [7]. In addition, direct RT-qPCR after long-term storage at 4 °C is not recommended as the detection rate decreases significantly over time.

Ct values of 91 SARS-CoV-2 positive samples analyzed in direct comparison by RT-qPCR using different primary materials and extracted RNA showed a significant correlation. As already indicated, not all samples were detected by both protocols. While only one sample was negative by RNA RT-qPCR, 17.6 % of previously positive tested SARS-CoV-2 samples could not be detected with the optimized direct RT-qPCR protocol. Detailed analyses have shown that a reduced sensitivity

of SARS-CoV-2 detection was predominantly associated with samples that had Ct values > 35 (52.6 %), as also seen in other studies [4,5,7,12]. Nevertheless, also a small number of samples with Ct values < 35 were negative for the detection of SARS-CoV-2 by direct RT-qPCR (4.2 %), which was most likely associated with long-term sample storage at 4 °C indicating a low stability of viral RNA without an extraction step. Only one sample in this study was RT-qPCR invalid (SARS-Cov-2 and internal control negative) indicating low drop off by PCR-inhibition of crude material.

Another major advantage of the PrimeDirect™ RT-qPCR is the very fast PCR cycling program (47 min in total) in combination with existing, accredited laboratory equipment. Isothermal detection of SARS-CoV-2 like LAMP (loop-mediated isothermal amplification) [13–16] are faster and can be done without real-time PCR cyclers, however examination of a color change is not an alternative for routine diagnostics, especially when there is no internal control. Compared to alternative high throughput crude specimen methods like LAMPSeq [16] or LamPORE (Oxford Nanopore Technologies) the direct RT-qPCR is one order of magnitude cheaper.

We found a very high SARS-CoV-2 detection rate of 95.8 % for Ct values < 35 by direct RT-qPCR and an overall detection rate of 81.3 %. These detection rates are comparable to other protocols for direct SARS-CoV-2 RT-qPCRs [4,5], however, our protocol is suitable for all kinds of respiratory material. Of note, several recent studies showed reduced or absence of infectivity in samples with a Ct value > 35 [17,18], therefore our detection rate is acceptable for mass screening or staff surveillance.

Taken together this protocol is an improvement to the currently available protocols and permits detection of SARS-CoV-2 from all kind of crude respiratory material. An internal control is mandatory to detect PCR inhibition and a pre-analytical RNA extraction should be considered for long stored samples. Notably, the extraction-free RT-qPCR is nearly as fast as isothermal amplification, but cheaper. The extraction-free RT-qPCR is therefore a cheap alternative to classical RNA-based RT-qPCR for example in mass screening when fresh samples are available.

Author contributions

Study concept, design and supervision (NL and AW), acquisition of samples (SS, MA, SH, OA), acquisition of data (NL and SS), analysis and interpretation of the data (NL and AW), statistical analyses and figure preparation (TS), drafting of the manuscript (NL, AW. and TS), critical revision of the manuscript (MA, SH, OA and JT), final approval of the manuscript (NL and AW).

Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jcv.2020.104579.

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