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# Assessment of Direct RT-qPCR for SARS-CoV-2 Detection by Multiplexed Assay

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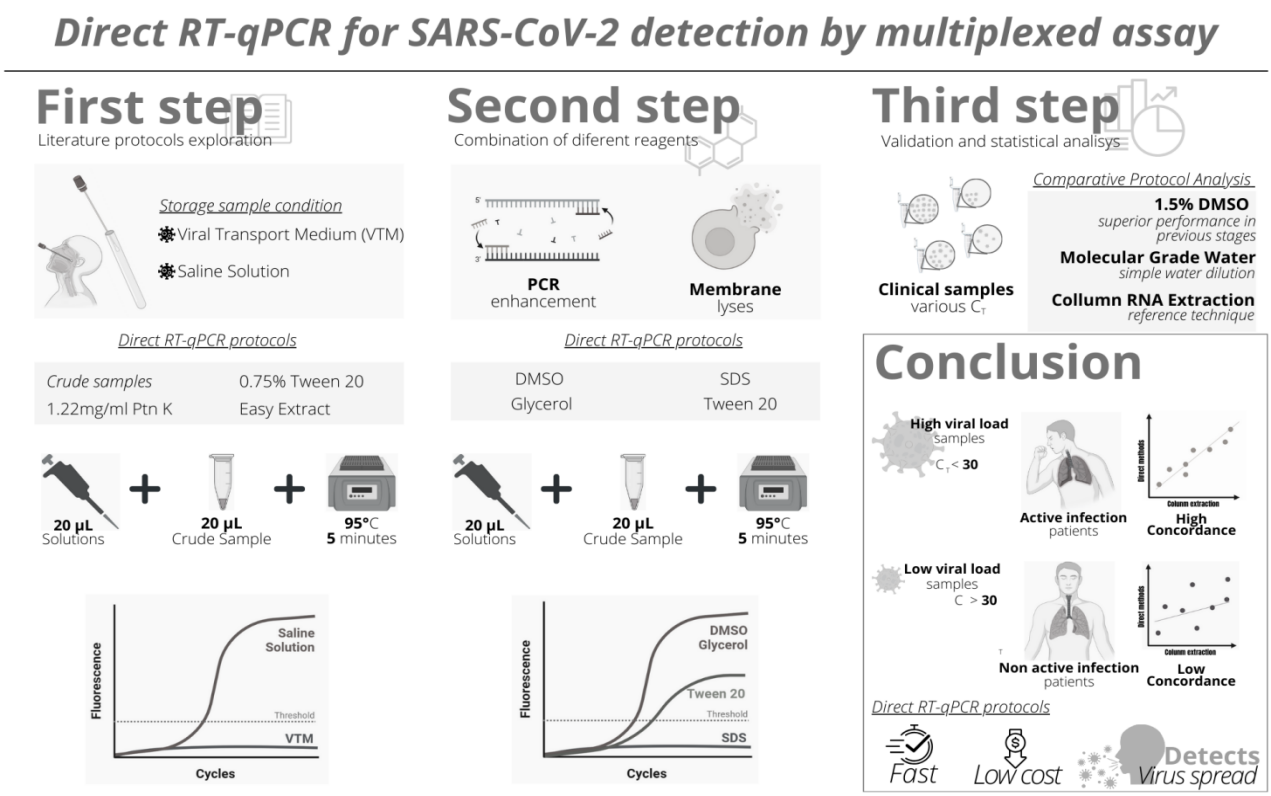
**HIGHLIGHTS**

- Sample transport medium interferes on direct RT-qPCR method.
- Sample dilution is a fast reliable method for SARS-CoV-2 detection.
- High concordance of direct RT-qPCR and reference extraction method ($C_T$<30).
- Allplex™ 2019-nCoV assay accurately detected SARS-CoV-2 in direct RT-qPCR.

**Abstract:** RNA extraction is usually required for molecular detection methods. On the other hand, this step implies time consumption and additional cost to laboratorial routine. This study evaluated the use of direct-RT-qPCR for SARS-CoV-2 detection compared to the gold standard extraction method. An exploratory stage was performed using DMSO, glycerol, Tween 20 and Easy Extract® DNA-RNA addition in a pool of highly positive clinical samples ($C_T$<25), which were subsequently tested by Allplex™ 2019-nCoV assay. Validation was performed with 54 SARS-CoV-2 positive and five negative clinical samples comparing three distinct conditions: (i) 1:1 water dilution, (ii) 1.5% DMSO dilution, or (iii) crude samples. Results of the exploratory stage showed Cycle Threshold ($C_T$) values very close to those obtained by the reference extraction step in high viral load samples. However, when lower viral load samples were analyzed, a slight concordance was observed compared to the extraction step. In the validation stage, it was observed that water or 1.5% DMSO dilution presented 97.3% of concordance in samples with $C_T$≤30, resulting in 97.4% of sensitivity and 0.83 Kappa coefficient. Sensitivity was reduced with samples $C_T$>30 and with crude samples. The methodology showed reproducibility with differents operators. These results reinforce the good performance of direct RT-qPCR methods in active SARS-CoV-2 infection, allowed cost and time reduction of diagnosis.

**Keywords:** COVID-19 Nucleic Acid Testing; SARS-CoV-2; Molecular Diagnosis; Sensitivity and Specificity.

**GRAPHICAL ABSTRACT**




## INTRODUCTION

RT-qPCR (real time quantitative RT-PCR) remains the worldwide gold standard molecular-based testing for SARS-CoV-2 and the most employed technique based on its high sensitivity and specificity. Technique reliability extends to molecular detection in low viral load samples, as those observed in asymptomatic patients or those after symptoms recovering [1]. Large-scale demand for molecular testing contributed to the development of new RT-qPCR assays for COVID-19 diagnostic emergence.

The development of multiplexed assays, like Allplex™ 2019-nCoV assay (Seegene Inc, Seoul, South Korea), provided the detection of multiple SARS-CoV-2 targets, with the combination of highly sensitive and specific genome regions, increasing the diagnosis accuracy [2]. Seegene assay was designed to target three viral genome regions: subgenus-specific Sarbecovirus envelope (E) gene, species-specifics SARS-CoV-2 nucleocapsid (N) and RNA-dependent RNA polymerase (RdRP) genes. This assay allows qualitative detection of SARS-CoV-2 at superior and inferior respiratory samples. An Internal Control (RP-V IC) composed of MS2 phage genome verifies all steps of the analysis process, including sample extraction, reverse transcription, and qPCR of each specimen [3].

During the pandemic breakthrough, the worldwide supply chain issues associated with shortage of human resources resulted in research efforts to perform direct RT-qPCR [3]. Skipping RNA extraction step significantly reducing reagent costs and testing time, mainly using samples with a minimum of processing, like heating, dilution, proteinase K treatment, and others. Previous studies have shown high performance in SARS-CoV-2 direct RT-qPCR [3-5]. However, each documented protocols presented a wide range of sensitivity and specificity values, even when used the same direct method.

The aim of this study was to assess the effectiveness of a rapid and cost-effective method for SARS-CoV-2 detection. Performance of direct RT-qPCR protocol in the Seegene assay was assessed comparing to the reference RNA extraction method of silica column.

## MATERIAL AND METHODS

### Clinical Samples

Nasopharyngeal swabs from patients between 3 to 10 days of symptom onset were collected, placed in sterile tubes containing 0.9% saline solution or viral transport medium (VTM), and stored at 4 °C until 24h prior laboratory analysis. Before diagnostic procedure and ultrafreezer storage, samples were divided into three aliquots. All samples were diagnosed using silica column extracted RNA by QIAamp® Viral RNA Mini Kit (Qiagen, Hilden, Germany) and RT-qPCR Allplex™ 2019-nCoV assay (Seegene).

The initial exploratory steps used pools of positive samples with $C_T$<25 (RpRd 24.48 ±0.615; E 22.44 ±0.475; 24.19 ±0.472), while the validation stage evaluated 54 SARS-CoV-2 positive and five negative clinical samples. Reproducibility was tested with eleven high $C_T$ value for N gene, ranging from 28.55 to 36.07.

### RNA extraction

The reference extraction method was performed using QIAamp® Viral RNA Mini Kit (Qiagen). Briefly, 140 µL of each nasopharyngeal sample were eluted on 60 µL AVE buffer and RT-qPCR kit Allplex™ 2019-nCov Assay extraction control RP-V IC (10 µL). The extraction process with each sample followed the manufacturer's instructions.

### Direct RT-qPCR methods

*Storage Sample Medium and Direct RT-qPCR Described Protocols Assessment*

Initially, a pool of positive clinical samples (N gene $C_T$<25) stored in saline solution or VTM was used to evaluate the influence of storage conditions in the performance of direct RT-qPCR protocol [3-7]. Before RT-qPCR reaction, 20 µL of crude samples were subjected to: (i) heating at 95°C for 5 min; or (ii) addition of 20 µL of three different solutions, followed by agitation and heating at 95°C for 5 min. The solutions assessed in this first step were (1) 1.22 mg/mL Proteinase K (Bioclin, Minas Gerais, Brazil) according Freppel et. al (2020) [3]; (2) 0.75% Tween 20 (Êxodo Científica, São Paulo, Brazil) solution; (3) Easy Extract® DNA-RNA kit (Interprise, São Paulo, Brazil). After heating, all preparations were cooled at 4°C for 5 min before RT-qPCR assay. The protocols were assessed in two independent experiments.

*Assessment of qPCR Efficiency with Enhancers Solutions*

As the next step, the same pool of positive nasopharyngeal samples was used to assess chemical components known to enhance qPCR efficiency or promote membrane lysis [4,8]. The protocol maintained the same proportion of 20 µL of positive crude samples mixed by agitation with 20 µL of each proposed solution, containing Tween 20, sodium sulfide (SDS), dimethyl sulfoxide (DMSO), glycerol or Proteinase K at different concentrations, followed by incubation at 95°C for 5 min compared to crude sample and silica-column extraction. The following direct RT-qPCR simple solutions were evaluated: DMSO (Êxodo Científica, São Paulo, Brazil), UltraPure™ glycerol (Invitrogen, Massachusetts, EUA), and Tween 20 (Êxodo Cientifica, São Paulo, Brazil) at concentrations of 0.75%, 1.5%, 3%, 6%; and SDS (Labsynth, São Paulo, Brazil) at 0.025%, 0.05%, 0.1%, and 1% concentrations. The extracted RNA from the same sample was used to assess the $C_T$ value variation. Two independent RT-qPCR analyses including all compared solutions were performed.

In third step, after simple solution assessment, more reliable components and concentrations were tested in a combined way, as described in Table 2. The extracted RNA from the same sample was used to assess the $C_T$ value variation. Two independent RT-qPCR analyses including all compared solutions were performed.

All previous outcomes were considered, and the most accurate direct RT-qPCR methods were selected to conduct a robust experiment with three replicates. Simple or combined solutions of DMSO and Glycerol, with or without heat treatment at 95°C for 5 min, were assessed, as described in Figure 1d.

*Validation stage*

All data from the first steps were considered, and based on exploratory outcomes, 54 positive and five negative clinical samples, were tested by direct RT-qPCR methods consisted of (i) 1.5% DMSO sample dilution at 1:1 was compared with (ii) molecular grade ultrapure water sample dilution 1:1; and (iii) crude sample application on RT-qPCR assay, without heating. These specimens were previously submitted to gold standard silica-column nucleic acid extraction followed by RT-qPCR Allplex™ 2019-nCov Assay. $C_T$ values

were utilized to categorize samples into different groups for the evaluation of the performance of direct RT-qPCR methods, including sensitivity, accuracy, and Kappa coefficient.

Outcomes observed in clinical samples with high $C_T$ values led to a second assessment using DMSO, water dilution and commercial Easy Extract® DNA-RNA solution in eleven high $C_T$ samples. Two negative samples were used in this assessment. Three independent replicates with three different operators were performed to evidence the reproducibility of direct RT-qPCR results.

*RT-qPCR*

SARS-CoV-2 RNA detection in clinical samples was carried out by RT-qPCR Allplex™ 2019-nCov Assay in CFX96 Touch Real-Time PCR Detection System (Bio-Rad, California, USA) following the manufacturer's instructions. Briefly, 8 µL of extracted, non-extracted (direct RT-qPCR methods), or crude sample template were combined to reagent mix in a total volume of 25 µL. The reaction conditions were as follows: reverse transcription at 50 °C for 20 min; cDNA pre-denaturation at 95 °C for 1 min; denaturation at 94 °C for 15 s, then 45 cycles of annealing and elongation (with fluorescence monitoring) at 58 °C for 30 s. RpRd, E, N viral gene and Internal Control (RP-V IC) $C_T \leq 40$ indicated a positive amplification and that greater than 40 represented a negative result. Clinical samples were considered positive for SARS-CoV-2 genome if there were: (i) amplification for all three genes; (ii) combined gene E and RpRd; (iii) E and N; (iv) only N or (v) RpRd. Positive samples for the RP-V IC and negative for viral genes were considered negative. Results were analyzed using software 2019-nCoV viewer (Seegene Inc Seoul, South Korea) according to the manufacturer's instructions.

RP-V IC internal control (1 µL) was also added to RT-qPCR to samples subjected to direct RT-qPCR methods. Positive control and no template control were assessed in all analyses.

*Statistics*

Direct RT-qPCR methods $C_T$ data were initially compared using Graphic Prism Software by Shapiro-Wilk normality test and subsequently submitted to parametric (One-way ANOVA test and pos-hoc Bonferroni) or non-parametric (Kruskal-Wallis) tests to determine statistical differences between methods. The level of significance was set at $P<0.05$.

Sensitivity, specificity, and accuracy of direct RT-qPCR processing methods considering silica-column nucleic acid extraction as reference method were calculated from MEDCALC online tool (https://www.medcalc.org/calc/diagnostic_test.php). Kappa coefficient was calculated by EPITOOLS online tool (https://epitools.ausvet.com.au/comparetwotestes). Kappa scale was considered as follow: Kappa < 0, no agreement; 0.00 – 0.20, slight agreement; 0.21 – 0.40, fair agreement; 0.41 – 0.60, moderate agreement; 0.61 – 0.80, substantial agreement; 0.81 – 1.00, almost perfect agreement [9].

*Ethical aspects*

The Ethical Review Committee from Federal University of Mato Grosso approved the use of clinical samples and anonymous information from notifications form in study protocol (CAAE 40625120.0.0000.5541/4.466.767).

## RESULTS

### Direct RT-qPCR methods

*Storage Sample Medium and Direct RT-qPCR Described Protocols Assessment*

Direct RT-qPCR methods presented no target amplification when samples were stored in VTM. Specimens stored in saline solution showed target genes and internal control (RP-V) amplification in all direct RT-qPCR methods, except for samples treated with proteinase K solution (1.22 mg/mL) (Table 1).

**Table 1**. Comparison of direct RT-qPCR methods results using samples storage in saline solution or VTM by Allplex™ 2019-nCov Assay.

| | Viral Transport Medium ($C_T$) | | | | Saline Solution ($C_T$) | | | |
|---|---|---|---|---|---|---|---|---|
| **Direct RT-qPCR Methods** | **RpRd** | **E** | **N** | **Result** | **RpRd** | **E** | **N** | **Result** |
| Crude sample + heat | ND | ND | ND | Invalid | 33.40 | ND | 29.03 | Positive |
| *1,22mg/mL* Ptn K* + heat | ND | ND | ND | Invalid | ND | ND | ND | Invalid |
| *0,75%* Tween 20 + heat | ND | ND | ND | Invalid | 32.87 | 31.57 | 28.88 | Positive |
| Easy Extract + heat | ND | ND | ND | Invalid | 25.75 | 20.47 | 20.64 | Positive |

CT – cycle of threshold; ND – not detected; Heat - 95°C for 5 minutes in a dry bath; Ptn K- Proteinase K. Invalid – No target and internal control amplification. *Protocol according to Freppel et. al (2020) [3]. $C_T$ values were the average of two independent RT-qPCR reactions.

*Efficiency of qPCR Enhancers Solutions*

Enhancer solutions with DMSO and glycerol at all concentrations tested separately showed similar direct RT-qPCR amplification results. Analysis of RpRd $C_T$ value evidenced a decrease of 1.80 cycle with 0.75% Glycerol + heating; the highest decrease of 2.99 cycles was observed with 0.75% DMSO + heating when compared to silica-column extraction. Otherwise, E and N genes required less cycles to surpass the threshold, the most favorable result was of 3.96 cycles less for the dilution with 3% Glycerol + heating (Table 2).

**Table 2.** RT-qPCR $C_T$ value variation in a pool of positive samples diluted in different combinations of DMSO, glycerol, Tween 20 solutions, and Easy Extract DNA-RNA solution.

| | | | | $C_T$ Direct Methods - $C_T$ Column* | | |
|---|---|---|---|---|---|---|
| **Direct RT-qPCR Methods$^{\Phi}$** | | | | **RpRd** | **E** | **N** |
| DMSO | 0.75% | | | 2.99 | -2.00 | -3.90 |
| DMSO | 1.50% | | | 2.53 | -2.39 | -3.87 |
| DMSO | 3.00% | | | 2.76 | -2.15 | -3.92 |
| DMSO | 6.00% | | | 1.98 | -1.27 | -2.01 |
| Glycerol | 0.75% | | | 1.80 | -2.97 | -3.77 |
| Glycerol | 1.50% | | | 3.01 | -2.75 | -3.37 |
| Glycerol | 3.00% | | | 1.90 | -2.61 | -3.96 |
| Glycerol | 6.00% | | | 2.06 | -2.27 | -3.73 |
| Tween 20 | 0.75% | | | 9.31 | 9.34 | 4.57 |
| Tween 20 | 1.50% | | | 10.07 | 9.87 | 5.33 |
| Tween 20 | 3.00% | | | 8.11 | 8.67 | 4.19 |
| Tween 20 | 6.00% | | | 9.11 | 9.22 | 4.75 |
| DMSO | 1.50% | Glycerol | 6.00% | 4.14 | -2.20 | -4.13 |
| DMSO | 1.50% | Glycerol | 0.75% | 2,58 | -3,48 | -5.16 |
| DMSO | 0.75% | Glycerol | 6.00% | 3.20 | -3.11 | -4.88 |
| DMSO | 0.75% | Glycerol | 0.75% | 2.52 | -3.17 | -5.09 |
| | | | Easy Extract | 1.51 | -2.11 | -4.69 |
| | | | Crude Sample | 11.34 | ND | 7.26 |

$C_T$ – cycle of threshold; ND - not detected. *Pool of samples was also submitted to RNA extraction by column method and used in RT-qPCR run. $C_T$ values were the mean of two independent RT-qPCR reactions using the same pool of positive samples.

Detergent solutions containing Tween 20 diminish the efficiency of RT-qPCR with an increase in the $C_T$ to all tested gene targets (Table 2), yet the sample still maintained positive test results ($C_T$<40). SDS solutions did not present any target or internal control amplification. Based on this outcome, Tween 20 and SDS were not employed in subsequent stages.

In the next step, solutions containing DMSO and Glycerol in different combinations, Easy Extract® DNA-RNA solution, and crude sample were assessed and compared with reference method. Detection of RpRd gene continued requiring more cycles to surpass threshold in direct methods, with higher CT values (4.14 cycles) in dilution with 1,5% DMSO and 6% Glycerol combined to heating. These combinations improved the sensitivity of E and N gene target detection in RT-qPCR, with the optimal result showing a reduction of 5.16 cycles to surpass the threshold to N gene, in samples diluted with a combination of 1.5% DMSO and 0.75% Glycerol solution and heating (Table 2).

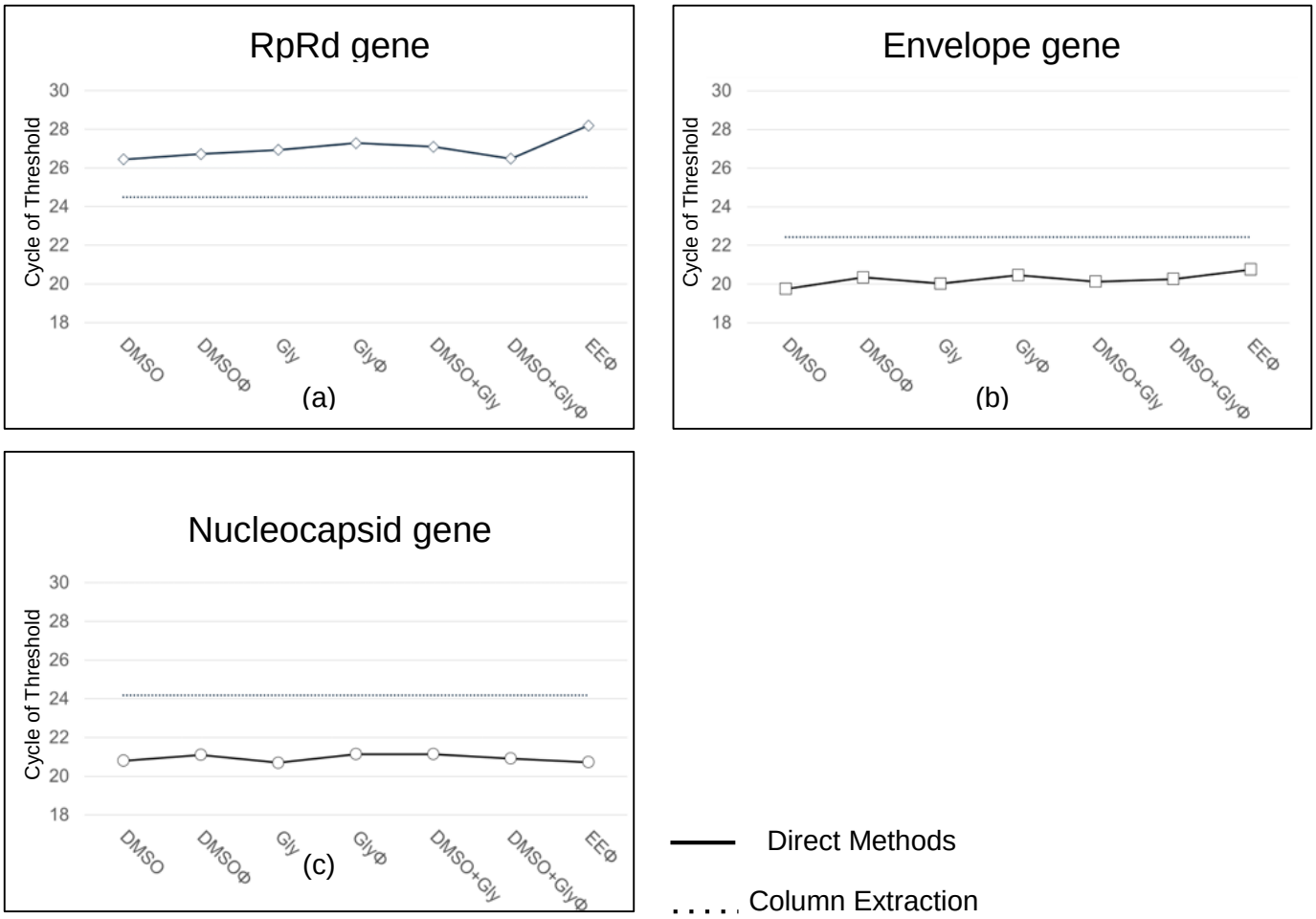
Commercialized Easy Extract® DNA-RNA solution exhibited most favorable results to detect RpRd target than previous solutions. This reagent showed an increase of 1.51 cycle of RpRd surpass threshold. Easy Extract® DNA-RNA and DMSO/Glycerol solutions presented similar results for N and E gene targets.

Direct crude sample showed a worse performance when compared with dilution methods, requiring 11.34 cycles more than silica-column extraction to surpass threshold for RpRd gene, 7.56 more cycles to N gene, and E gene was not detected, despite these data represent positive SARS-CoV-2 Seegene assay result.

Previous results with simple or combined DMSO and Glycerol enhancer solutions were evaluated, with or without heat treatment, and showed a superior performance on direct RT-qPCR methods when compared to silica-column reference method for E and N gene target detection in three-replicate experiments. Statistically significant decreased E gene $C_T$ values were observed with sample dilution in 1.5% DMSO and combined 0.75% DMSO + 0.75% glycerol solutions without heating. Decreases in N gene $C_T$ values were significant when samples were diluted in 1.5% DMSO, 6% Glycerol, or commercial Easy Extract® DNA-RNA solutions (Figure 1).

Corroborating with previous assessments, direct RT-qPCR methods with evaluated solutions decreased the efficiency of RpRd gene detection. Significant higher $C_T$ values were observed when samples were diluted in 1.5% DMSO, or 6% Glycerol and heated, or in 6% Glycerol dilution only. Analysis showed that heating of

diluted samples did not present a significant impact on $C_T$ value of all assessed gene targets, combined or not to enhancer solutions, by direct RT-qPCR (Figure 1).




| Direct RT-qPCR Methods | | | | | RpRd | E | N |
|---|---|---|---|---|---|---|---|
| DMSO | 1.5% | | | | 26.44 (±0.23) | 19.75 (±0.13)$^{d,e}$ | 20.80 (±0.06)$^{f}$ |
| DMSO | 1.5% | Φ | | | 26.72 (±0.35)$^{a}$ | 20.35 (±0.25) | 21.10 (±0.20) |
| Glycerol | 6% | | | | 26.94 (±0.25)$^{b}$ | 20.02 (±0.20) | 20.70 (±0.14)$^{g}$ |
| Glycerol | 6% | Φ | | | 27.29 (±0.26)$^{c}$ | 20.46 (±0.26) | 21.14 (±0.41) |
| DMSO | 1.5% | Glycerol | 6% | | 26.66 (±0.32) | 19.73 (±0.11) | 20.72 (±0.14) |
| DMSO | 1.5% | Glycerol | 6% | Φ | 27.03 (±0.38) | 20.46 (±0.37) | 21.22 (±0.42) |
| DMSO | 0.75% | Glycerol | 0.75% | | 27.10 (±0.27) | 20.13 (±0.17) | 20.91 (±0.10) |
| DMSO | 0.75% | Glycerol | 0.75% | Φ | 26.48 (±0.17) | 20.26 (±0.27) | 21.05 (±0.24) |
| | | | Easy Extract | | 28.20 (±2.81) | 20.75 (±0.59) | 20.72 (±0.03)$^{h}$ |
| | | | Column extraction | | 24.48 (±0.61)$^{a,b,c}$ | 22.44 (±0.47)$^{d,e}$ | 24.19 (±0.47)$^{f,g,h}$ |

**(d)**

**Figure 1.** Direct RT-qPCR methods based on sample dilution with dimethyl sulfoxide (DMSO), glycerol (Gly) and Easy Extract® DNA-RNA (EE) solutions compared with silica-column extraction. (a) Graphic representation of $C_T$ value of RpRd, (b) E and (c) N gene targets in the same pool of positive samples across the direct RT-qPCR methods. Φ indicates 95°C heating for 5 min. (d) Table contents target $C_T$ values and standard deviation. $^{a-h}$ Statistical difference between methods (P<0.001).

## Validation stage

Based on the direct RT-qPCR outcomes observed with the pool of positive clinical samples (CT<25), the simplest and fastest method (1.5% DMSO dilution 1:1 without heat step), was chosen to assess fifty-four positive and five negative clinical samples previously extracted by silica-column and diagnosed by RT-qPCR

Allplex™ 2019-nCoV assay. Samples diluted in pure molecular grade water (1:1) were considered to assess the effect of simple dilution on outcomes. Crude samples (8 µL) were also tested in direct RT-qPCR.

Analysis showed high sensitivity (97.44%) and almost perfect agreement (Kappa = 0.89) performance in both 1:1 dilution in 1.5% DMSO and water, considering thirty-nine samples with N gene $C_T \leq 30$ (Table 3). When fifteen samples with $C_T > 30$ were included in the analysis, sensitivity decreased for both 1.5% DMSO (75.93%) and water (79.63%) 1:1 dilution, accompanied by kappa coefficient (fair agreement). Direct crude samples exhibited low sensitivity and slight agreement in all $C_T$ values. All tested direct RT-qPCR methods showed 100% of specificity.

**Table 3.** Direct RT-qPCR methods sensitivity, accuracy, and concordance considering grouped samples with different N gene CT values.

| N Gene $C_T$ | Direct RT-qPCR Methods | TP (n) | FN (n) | SNS (%) | Acc (%) | K |
|---|---|---|---|---|---|---|
| *Samples* **$C_T \leq 20$** | 1.5% DMSO 1:1 dilution | 20 | 0 | 100.00 | 100.00 | 1.00 |
| | Water 1:1 dilution | 20 | 0 | 100.00 | 100.00 | 1.00 |
| | Crude sample | 9 | 11 | 45.00 | 56.00 | 0.24 |
| *All samples* **$C_T \leq 30$** | 1.5% DMSO 1:1 dilution | 38 | 1 | 97.44 | 97.73 | 0.89 |
| | Water 1:1 dilution | 38 | 1 | 97.44 | 97.73 | 0.89 |
| | Crude sample | 19 | 20 | 48.72 | 54.55 | 0.17 |
| *All samples* **$C_T \leq 36$** | 1.5% DMSO 1:1 dilution | 41 | 13 | 75.93 | 77.97 | 0.34 |
| | Water 1:1 dilution | 43 | 11 | 79.63 | 81.36 | 0.39 |
| | Crude sample | 19 | 35 | 35.19 | 40.68 | 0.08 |

**TP** – True positive. **FN –** False Negative. **SNS –** Sensitivity. **Acc –** Accuracy. **K –** Kappa Coefficient.

$C_T$ values of N gene target showed higher concordance between direct methods and reference silica-column method, with a determination coefficient ($R^2$) of 0.83 (1.5% DMSO) and 0.81 (Water dilution). E gene presented the second-highest determination coefficient (0.77) with 1.5% DMSO. RpRd $C_T$ values showed low determination coefficients in both direct methods tested (Figure 2).

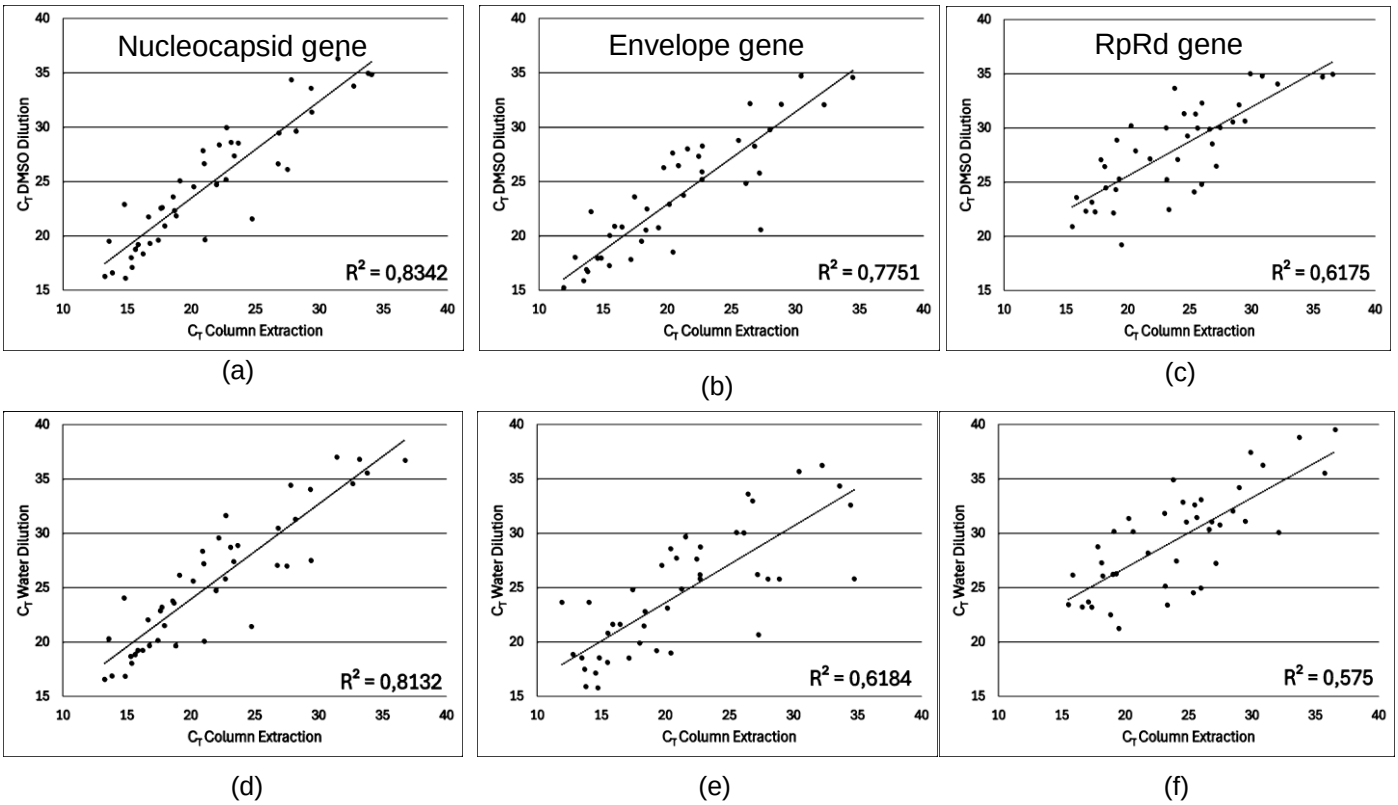



**Figure 2.** Linear regression plot of target genes $C_T$ values in direct RT-qPCR methods and reference sample RNA extraction. Gene targets $C_T$ values with 1.5% DMSO dilution at 1:1(a,b,c). Gene targets $C_T$ values at water 1:1 dilution (d,e,f).

According to the lower efficiency of direct RT-qPCR detection for samples with $C_T$>30 for the three evaluated genes, the reproducibility of results was assessed with different operators. It was observed a 100% of concordance until $C_T$ 32.17 (Table 4) between direct methods and operators. No detection was observed in samples with $C_T$≥34.07 on gold standard silica-column method for the three operators.

**Table 4.** Reproducibility of direct RT-qPCR methods performed by three different operators using samples with high $C_T$ values on gold standard silica-column method.

| Sample | Operator 1 | | | Operator 2 | | | Operator 3 | | |
|---|---|---|---|---|---|---|---|---|---|
| N gene $C_T$ | Water | 1,5% DMSO | Easy Extract $^{\Phi}$ | Water | 1,5% DMSO | Easy Extract $^{\Phi}$ | Water | 1,5% DMSO | Easy Extract $^{\Phi}$ |
| 28.55 | P | P | P | P | P | P | P | P | P |
| 31.38 | P | P | P | P | P | P | P | P | P |
| 31.65 | P | P | P | P | P | P | P | P | P |
| 31.83 | P | P | P | P | P | P | P | P | P |
| 32.17 | P | P | P | P | P | P | P | P | P |
| 32.62 | ND | P | P | ND | P | P | P | P | P |
| 32.74 | P | P | P | P | P | P | P | P | P |
| 34.07 | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| 34.39 | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| 35.00 | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| 36.07 | ND | ND | ND | ND | ND | ND | ND | ND | ND |

P – positive. ND – not detected. $^{\Phi}$95°C heating for 5 min.

## DISCUSSION

COVID-19 pandemic context has required the improvement of simpler, faster, and cheaper diagnosis tests to elicit an efficient patient management and strategies to control virus spread. Fast assay techniques based on antigen or antibody detection may present low specificity [1], while the advancement of RT-qPCR technique has been highlighted recently. Several studies present evidence of good performance of simplified sample preparation methods prior to RT-qPCR assay [10,11].

Initially, assays were performed to appraise direct RT-qPCR methods already described, with the purpose to choose the superior performance method for the validation step. The interference of stored medium in samples was assessed, considering saline solution and VTM. Specimen storage in VTM showed no amplification for any target, either after silica-column extraction or direct crude sample application on RT-qPCR. This inhibitory effect corroborates previous studies [5,13-15] suggesting that VTM contains RT-qPCR inhibitors. Despite that, several controversial outcomes regarding SARS-CoV-2 amplification of samples stored in VTM have also been described. Some direct RT-qPCR protocols reached successful results in VTM stored samples, using enzymes resistant to RT-qPCR inhibitors [5,8,16]. Freppel and coauthors [3] suggested that a higher dilution might be necessary for detection on specimens stored in VTM.

In contrast to earlier findings, our initial assessment of the proteinase K direct method showed no target amplification even for internal control, suggesting an inhibitory effect [3]. Proteinase K was not maintained in the next steps mainly because it required an extra step of 50°C for 10 min, postponing diagnosis. Furthermore, previous studies of proteinase K method demonstrated that sensitivity and specificity were like those obtained by more simplified direct methods proposed in our study [3,6,17-19].

Reported protocols evaluated the combination of heating and mild detergents (i.e. Tween-20) for lysis of virus and human cells to detect SARS-CoV-2 evidencing no impact in RT-qPCR assay [4,8]. Even with positives results in this study, samples treated with Tween-20 solutions showed an increase of $C_T$ values, when compared to those obtained with silica-column method (Table 2).

Simple or combined solutions containing DMSO and glycerol showed promising outcomes. Samples with low target genes $C_T$ presented results comparable to those obtained with the reference silica-column extraction method and with the commercial solution Easy Extract® DNA-RNA (Table 3). Considering previous exploratory stage results, a validation stage was performed to assess clinical samples. Fifty-four positive samples formerly diagnosed by reference method were stratified by gene N $C_T$ value for detailed analysis: $C_T \leq 20$, $20 < C_T \leq 30$, and $C_T \leq 36$ (Table 3). Categorized clinical samples were submitted to 1.5% DMSO and water dilution at 1:1 to evaluated diagnose performance. Results showed 100% of sensitivity for samples with $C_T < 20$, while decreased sensitivity was observed with $C_T$ increases (Table 3). The number of false negatives observed in $C_T > 20$ samples by direct methods reinforce a loss of accuracy in low viral load specimens.

In consideration of the fragility of direct RT-qPCR method for low viral load samples ($C_T > 20$), it was proposed the assessment of technique reproducibility testing samples $C_T$ ranging from 28.55 to 36.07 obtained by reference diagnosis. Results corroborated to previous observations, increasing false negative results along $C_T$ value (Table 4). Published data supported our results demonstrating a decrease of sensitivity in direct methods when samples had a high target $C_T$ value on RT-qPCR after standard silica-column method. Domnich and coauthors [20] and Beltrán-Pavez and coauthors [21] observed a sensitivity of 90% decrease to about 19-40% in samples with $C_T > 30$; Cameron and coauthors [22] and Schoeber and coauthors [23] described this effect in samples $C_T > 35$; and Zou and coauthors [24] in $C_T > 37$. The main reason for this sensitivity loss is due to column extraction employment which concentrates viral RNA (~2,3 times), while direct methods may dilute samples. However, it is important to highlight that low viral load cases (high $C_T$ value) have been described exhibiting lower infectivity [25] and have been related with late stages of infection. SARS-CoV-2 testing have been recommended when COVID-19 symptoms are evident, probably presenting high viral loads [26].

As direct RT-qPCR methods, 1.5% DMSO and water dilution were chosen in this study and showed comparable results, suggesting a dilution effect of RT-qPCR reaction inhibitors. Such outcome was reinforced when samples were diluted in water (1:1) showing $C_T$ values next to that obtained in 1.5% DMSO 1:1 dilution (Figure 2). In contrast, direct application of eight microliter of crude samples presented false negative results independently of $C_T$ value (Table 3). A minor volume of direct sample application was not tested in this study, although Bruce and coauthors [27] showed that amplification is possible using less than three microliters of direct sample in AgPath-ID One-Step RT-qPCR kit (Life Technologies) with an increase of sensitivity.

Reliable performance of direct RT-qPCR method was reached considering N gene (Figure 2). This outcome supports evidence from previous observations [3,6,20], which state that N gene is the most sensitive target for SARS-CoV-2 diagnosis, considering its presence in all subgenomic mRNAs, while E and RdRP are less represented in samples [28].

## CONCLUSION

Many studies, with diverse strategies of direct methods showed evidence of a reliable performance in RT-qPCR assay for SARS-CoV-2 detection. However, local validation is required to achieve diagnostic implementation, considering different sensitivity and specificity values observed for different published protocols. Overall, documented data for direct RT-qPCR method strengthens the idea that successful performance depends on the efficient combination of samples transport solution and the detection assay.

Considering the literature database and adapting protocols to our laboratorial structure, we conclude that simple dilution of specimens in nuclease free water reach an adequate sensitivity, mainly in samples represented by individuals with high transmission rate. Our proposed direct RT-qPCR methods provided results in a shorter time, low cost, thereby speeding up clinical diagnostics. Indeed, the average processing time in our protocol was 5 min per sample. The main limitation of this study was the evaluation of only one RT-qPCR assay. A superior performance could be reached testing other enzymes and target regions, maintaining N gene detection. Robust CDC protocol based on N1 and N2 genome region could be used for sensitivity comparation. This study findings contribute to current data of direct methods performance and could guide selection of RT-qPCR detection systems providing a template for comparison with alternative protocols.

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