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Simple RNA Extraction and Viral Inactivation Methods for Detecting SARS-CoV-2 in Nasopharyngeal Swabs from Clinically Suspected COVID-19 Patients

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ABSTRACT

In December 2019, pneumonia cases of unknown origin were identified in individuals working or residing near the Huanan Seafood Market in Wuhan, China. Sequencing of respiratory samples revealed a novel coronavirus, initially named 2019-nCoV, now known as SARS-CoV-2. The viral genome encodes key proteins including those involved in replication (Rep 1a and Rep 1b) and structural proteins (S, E, M, N). Real-time PCR (RT-PCR) emerged as the gold standard for COVID-19 diagnosis, amplifying specific regions of the virus's genetic material to estimate viral load using cycle threshold (Ct) values. However, RT-PCR's accuracy is influenced by factors such as sample collection, storage, and timing of testing. This process includes mRNA amplification via extraction of viral RNA from respiratory samples using commercial kits. RNA extraction, a crucial step, significantly impacts the accuracy of subsequent analyses, with growing demands for reliable and automated extraction kits. The quality of RNA is paramount, but accessibility remains a challenge in lower-resource settings. This study aims to optimize a simple RNA extraction and viral inactivation method, evaluating factors such as freezing, agitation, and protease treatment to enhance the sensitivity of SARS-CoV-2 RNA detection in nasopharyngeal swabs. The findings propose a simplified alternative for SARS-CoV-2 RNA extraction and purification.

Keywords: SARS-CoV-2; RT-PCR; RNA Extraction; Viral Inactivation; Diagnostics; Cycle Threshold; Nasopharyngeal Swabs; Viral Load; COVID-19; RNA Inactivation

Introduction

In December 2019, several individuals who worked or lived at the Huanan Seafood Market in Wuhan, Hubei Province, China, developed pneumonia of unknown origin [1,2]. Sequencing analysis of respiratory samples identified a new coronavirus, initially named 2019 novel coronavirus (2019- nCoV) and now known as SARS-CoV-2 [3,4]. The viral genome encodes several essential proteins, including the Rep 1a and Rep 1b regions involved in viral replication, and the S, E, M, and N regions, which code for structural proteins [5,6]. Real-time reverse transcription-polymerase chain reaction (real-time PCR or RT-PCR) has become the gold standard for diagnosing COVID-19 [7,8]. The RT-PCR process amplifies well-defined regions of the virus's genetic material, multiplying those thousands of times. This amplification generates sufficient material to be identified, resulting in cycle threshold (Ct) values [9,10]. Ct values in COVID-19 RT-PCR tests provide an estimate of the viral load in biological samples [11,12]. However, the RT-PCR method has certain limitations, and its accuracy depends on variables such as proper sample collection and storage, the timing of

the test relative to the infection window, internal validation, and cases where the absence of genetic material amplification does not necessarily exclude infection [12-14]. For viruses like SARS-CoV-2, mRNA amplification is performed by collecting respiratory samples from the person being tested, followed by the extraction of genetic material using specific commercial kits [7,8].

The RT-PCR process involves several steps, including the extraction of viral RNA from the clinical samples collected [9,10]. The first step, RNA extraction, is critical for COVID-19 detection as it determines the quality of RNA used in subsequent analysis. Throughout the pandemic, there has been a growing demand for reliable commercial kits designed for automated SARS-CoV-2 RNA extraction [8,15]. The quality and quantity of RNA are key factors as they directly influence the accuracy of gene expression analysis and other downstream RNA- based applications [16,17]. High-quality RNA is essential for reliable results, but the COVID-19 emergency has highlighted the inaccessibility of many advanced kits and equipment in low-complexity laboratories and developing countries [18,19]. The goal of this study is to optimize a simple RNA extraction and viral inactivation method for use in SARS-CoV-2 diagnostics [20-23]. In this study, we evaluated the influence of various factors — such as freezing, agitation, and protease treatment — on the sensitivity of SARS-CoV-2 RNA detection in clinical samples (nasopharyngeal swabs [NPS] from clinically suspected COVID-19 patients) and ultimately aimed to propose a simple alternative method for viral RNA extraction and purification [24-30].

Materials and Methods

PICO Strategy

The PICO strategy was employed to simplify the research question. This methodological tool is widely used to formulate research questions clearly and objectively, particularly in the fields of health and evidence-based medicine [31-33]. The acronym PICO stands for four main elements: P (Patient or Problem), I (Intervention), C (Comparison), and O (Outcome). In this study, the element (P) was defined as patients with clinical suspicion of COVID-19 at the Local Laboratory; (I) represented samples that underwent the SARS-CoV-2 inactivation process; (C) referred to samples that underwent the process of genetic material extraction for the SARS-CoV-2 virus; and (O) aimed to assess the impact of this inactivation on RT-PCR results. The research question established was: “Does the virus inactivation method positively influence the diagnosis of COVID-19 using the RT-PCR technique?” This question guided the methods used in this research. Five laboratory tests were conducted (Figure 1).

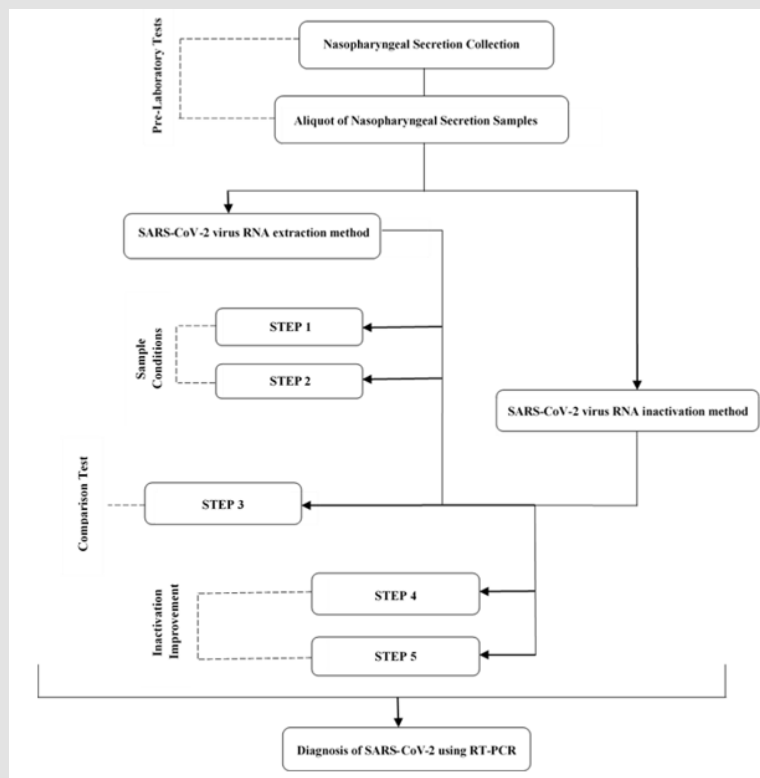

Figure 1: Flowchart of the Laboratory Testing Procedure.

Pre-Laboratory Tests

Nasopharyngeal secretion collection is a medical procedure used to obtain samples from the upper part of the throat behind the nose (nasopharynx). It is commonly performed for diagnostic purposes, especially to detect respiratory infections such as SARS-CoV-2, influenza, and other pathogens [34-36]. Samples of nasopharyngeal secretions were collected using sterile rayon swabs to obtain secretions from both nostrils of patients with suspected signs for COVID-19. After collection, the swab was immediately placed into a 10 mL polypropylene conical tube containing 2 mL of 0.9% sodium chloride, following the established protocol for routine use at the Local Laboratory [37-40]. The swab samples were rinsed in approximately 2 mL of 0.9% sodium chloride, vortexed, and 200 µL aliquots of the samples were transferred into 1.5 mL Eppendorf tubes. Aliquots were prepared at all stages of the laboratory tests to ensure sufficient material was available for each step of the testing process [41-45].

Diagnostic Testing for COVID-19

Patients with suspected signs for COVID-19 are tested for the presence of SARS-CoV-2 using real-time reverse transcriptase polymerase chain reaction (RT-PCR) from nasopharyngeal secretion samples [8,28,45,46]. Swab samples collected must be promptly transported to designated laboratories, where RNA is properly extracted. Detection is performed using RT-PCR with primers and probes specific to the target sequences. In tests 01, 02, and 03, after arriving at the laboratory, the viral material was extracted using the local laboratory's gold standard method, the "Pure Link Viral RNA/DNA Mini Kit (Cat. No. 12280050, Invitrogen, USA)," following the guidelines provided in the manufacturer's manual [31,47].

Sample Conditions

Step 1 (Alternative Procedure for Storaging Samples): during routine practice at the local laboratory, nasopharyngeal swab samples were processed immediately upon arrival and kept at room temperature throughout all processing stages. These samples were stored in a refrigerator only during the RT-PCR amplification and analysis period and were reused only in cases of experimental failure. Based on the established knowledge that freezing at specific temperatures halts cellular metabolic pathways and preserves cells and tissues for extended periods, prior freezing was tested as an alternative storage method for these samples. Two aliquots of nasopharyngeal swab samples were collected from 30 patients confirmed positive for COVID-19. The first aliquots were processed upon arrival at the local laboratory (in accordance with routine procedures), while the remaining aliquots were subjected to prior freezing (~-18 °C) for 30 minutes.

Step 2 (Sample Shelf Life Evalutaion): Due to the high turnover of nasopharyngeal swab samples during the COVID-19 pandemic, the Local Laboratory lacked the physical infrastructure for prolonged storage. As a result, after the test results were released, the samples were discarded and incinerated. Based on the premise that other high-complexity laboratories routinely store samples for extended periods, we evaluated the shelf life of samples from 15 patients confirmed positive for COVID-19 over a period of seven days. Four aliquots were taken from each sample: the first aliquot was processed 30 minutes after prior freezing (~-18°C) for 30 minutes on day 0 (D0), the second aliquot was processed on day 3 (D3), the third on day 5 (D5), and the fourth on day 7 (D7).

Comparison Test

Step 3 (Virus inactivation procedure): At the onset of the COVID-19 pandemic, the primary challenge to implementing large-scale RT-PCR testing in laboratory routines was the difficulty in identifying an RNA extraction process that was efficient, low-cost, fast, and biosafety compliant. Based on this challenge, in experiment No. 3, the SARS-CoV-2 RNA extraction process was replaced with a virus inactivation protocol involving heating to 95 °C for 15 minutes in a Dry Water Bath NT 362 – Dry Blo. Two aliquots of nasopharyngeal swab samples were taken from 300 patients presenting clinical symptoms of COVID-19 but without a confirmed diagnosis. The first aliquot was frozen for 30 minutes upon arrival at the Local Laboratory. After freezing, 25 µL of "Proteinase K" (20 mg/mL, Invitrogen™) was added and vortexed. The aliquot was then heated to 95°C for 15 minutes in the dry water bath. The second aliquot was similarly frozen (~-18 °C) for 30 minutes upon arrival, but RNA extraction was conducted using the standard method routinely applied at the laboratory.

Inactivation Improvement

Step 4 (Assessing the Optimal Amount of Proteinase K): After validating the inactivation process, in experiment No. 4, we determined the optimal volume of Proteinase K to add to the samples. This approach aims to minimize costs and prevent unnecessary enzyme waste. Four aliquots of nasopharyngeal swab samples were collected from 15 patients confirmed positive for COVID-19. All aliquots were frozen (~-18 °C) for 30 minutes upon arrival at the Local Laboratory. In the first aliquot, 25 µL of Proteinase K was added; in the second, 15 µL; in the third, 10 µL; and in the fourth, 5 µL. After enzyme addition, all aliquots were subjected to 95 °C for 15 minutes in a Dry Water Bath NT 362 – DryBlo.

Step 5 (Evaluation of Samples Shaking Effects): In this final experiment, we chose to test the use of a thermoblock with a shaker (MTC-100 Thermo Shaker Incubator - Hangzhou Mil Instruments Co. Ltda) to determine whether shaking the samples during the 15 minutes at 95 °C helps achieve better homogenization of the samples in the inactivation process. Two aliquots of nasopharyngeal swabs were collected from 30 patients confirmed positive for COVID-19. These aliquots were frozen (~-18 °C) for 30 minutes upon arrival at the Local Laboratory, and 25 µL of Proteinase K was added. One aliquot was subjected to 95 °C for 15 minutes in a Dry Water Bath NT 362 – Dry-

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Blo, while the other aliquot was subjected to 95 °C for 15 minutes in a thermoblock with shaker (MTC- 100 Thermo Shaker Incubator - Hangzhou Mil Instruments Co. Ltda).

Diagnosis of SARS-CoV-2 using Real-Time PCR

For the diagnosis of SARS-CoV-2, all samples included in the study used the "VIASURE SARS-CoV-2 Real-Time PCR Detection Kit" with Ref. VS- NCO236 and VS-NCO272, following the guidelines in the manufacturer's manual (CerTest Biotec). The ORF1ab region of SARS-CoV-2 plays a crucial role in COVID-19 diagnosis using the RT-qPCR technique, as it is one of the main target regions for virus detection. ORF1ab is a viral genome sequence that encodes non-structural proteins essential for viral replication and infection, such as RNA-dependent RNA polymerase (RdRp) and viral protease. These proteins are vital for viral replication, making the ORF1ab region a reliable target for detecting SARS-CoV-2 in the body. Thus, the cycle threshold (Ct) value of the ORF1ab gene was used to determine the detection result, with a Ct value greater than or equal to 30 defined as negative, and below 30 as positive. The Ct value of the N gene was analyzed following the initial analysis of the ORF1ab gene. The Qiagen/Corbett Rotor-Gene® instruments, along with accessories compatible with Real-Time PCR, were used. The analysis of Real-Time PCR data generated by the Rotor-Gene thermocycler was performed using the "Q-Rex" software, developed by QIAGEN.

Results

The results were categorized and presented according to the respective tests.

Step 1

A sample from 30 patients known to be positive for COVID-19 was processed immediately upon arrival at the Local Laboratory at room temperature. All these samples showed a delay in the amplification of the ORF1ab and N genes compared to the amplification results of the same genes from samples that had been previously frozen (~-18 ºC) for 30 minutes (Figure 2) (Supplementary Table 1). For the ORF1ab gene, the average Ct values were 25.0 and 23.0, with standard deviations (SD) of 2.51 and 2.41 cycles, respectively (p-value = 0.0008917). For the N gene, the average Ct values were 26.4 and 24.8, with SD values of 2.17 and 2.49 cycles, respectively (p-value = 0.002944) (Table 1).

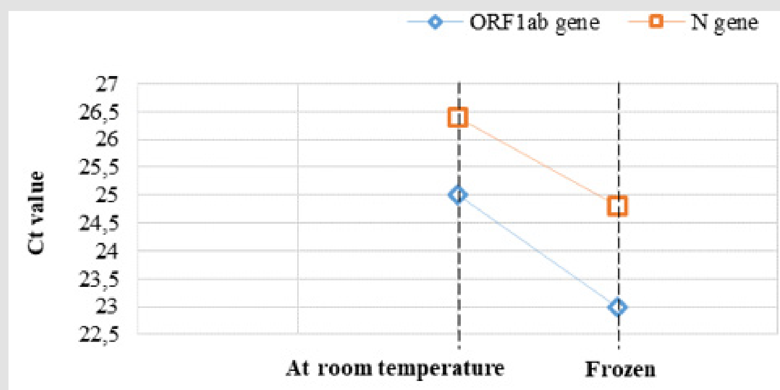

Figure 2: Comparative reverse transcription real-time polymerase chain reaction (RT-qPCR) results in the comparison of two preconditioning temperatures ("At room temperature" and "Frozen") of a total n of 30 nasopharyngeal swab samples from patients known to be positive for COVID-19. The data is shown in an illustrative way, to demonstrate in an average format the difference in the cycle threshold (Ct) values between the results found.

Table 1: Comparative reverse transcription real-time polymerase chain reaction (RT-qPCR) results were obtained by comparing two preconditioning temperatures ("room temperature" and "frozen") for a total of 30 nasopharyngeal swab samples from COVID-19-positive patients. The data are presented as the mean ± standard deviation (SD) of cycle threshold (Ct) values for the ORF1ab and N genes of SARS-CoV-2, along with the p-value comparing the average results obtained.

At Room Temperature			Frozen		
	Mean (Ct Value)	Standard deviation (Ct value)	mean (Ct value)	Standard deviation (Ct value)	
ORF lab gene	25,0	2,51	23,0	2,41	p-value=0,0008917
N gene	26,4	2,17	24,8	2,49	p-value=0,002944

Supplementary Table 1: Cycle threshold (Ct) results for the amplification of the ORF1ab and N genes of the SARS-CoV-2 virus from all 30 (thirty) nasopharyngeal secretion samples of COVID-19 positive patients, comparing the results of two previous conditions of these samples (at room temperature and frozen) – Continued.

Sample Condition							
		At room temperature				Frozen	
	Sample Number	ORF1ab gene (Ct value)	N gene (Ct value)	Result	ORF1ab gene (Ct value)	N gene (Ct value)	Result
Total number of samples: 30 (COVID-19 positive)	1	25,9	26,8	Positive*	23,3	25,2	Positive
	2	24,2	25,9	Positive	21,1	23,9	Positive
	3	27,3	28,2	Positive	24,4	26,8	Positive
	4	27,3	29	Positive	24,1	25,9	Positive
	5	25,2	26,7	Positive	23,7	25,1	Positive
	6	26,8	27,5	Positive	25,3	27,3	Positive
	7	27,5	28,9	Positive	26,3	28,2	Positive
	8	23,1	25,3	Positive	19,7	21,5	Positive
	9	24,5	26,1	Positive	23	25,1	Positive
	10	22,8	24,7	Positive	21,4	23,7	Positive
	11	24,6	25,9	Positive	23,3	25,9	Positive
	12	21,4	24,7	Positive	19,3	20,3	Positive
	13	19,6	21	Positive	18,7	21	Positive
	14	22,9	24,2	Positive	21,4	23,7	Positive
	15	27,6	29,4	Positive	25,5	27,4	Positive
	16	29,8	30,4	Positive	27,6	29,7	Positive
	17	27,6	28,4	Positive	25,3	27,5	Positive
	18	23,8	24,7	Positive	21,6	23,4	Positive
	19	25,7	26,9	Positive	23,2	23,1	Positive
	20	23,6	25,7	Positive	21	23	Positive
	21	26,4	26,9	Positive	25,3	26,9	Positive
	22	25,2	26,3	Positive	23,7	25,1	Positive
	23	29,9	30,5	Positive	26,9	29,3	Positive
	24	24,3	25,6	Positive	22,8	24,1	Positive
	25	25,9	26,8	Positive	23,9	25,4	Positive
	26	24,5	25,7	Positive	22,1	24,1	Positive
	27	25,9	27,1	Positive	24,8	25,7	Positive
	28	23,9	24,6	Positive	22,9	23,5	Positive
	29	23,1	24,8	Positive	21,5	23,8	Positive
	30	19,3	21,9	Positive	17,6	19,2	Positive

Note: "Positive*" = positive for COVID-19.

Step 2

Four aliquots were taken from 15 patients known to be positive for COVID-19. Each aliquot was previously frozen (~-18 ºC) and processed at different time points (1st day, 3rd day, 5th day, and 7th day). An increase in the delay in amplification of the ORF1ab and N genes was observed as the days progressed (Figure 3) (Supplementary Table 2). For the ORF1ab gene, the average Ct values were 21.00, 23.66, 31.49, and Not Detected, with SD values of 2.74, 2.87, 0.95, and invalid cycles, respectively. When comparing the 1st and 3rd days, the result was statistically significant with a p-value of 0.0003615, and when comparing the 3rd and 5th days, the p-value was 0.03052. For the N gene, the average Ct values were 22.73, 24.99, 33.23, and Not Detected, with SD values of 2.63, 2.56, 1.23, and invalid cycles, respectively. When comparing the 1st and 3rd days, the result was statistically significant with a p-value of 0.0003606, and when comparing the 3rd and 5th days, the p-value was 0.03052 (Table 2).

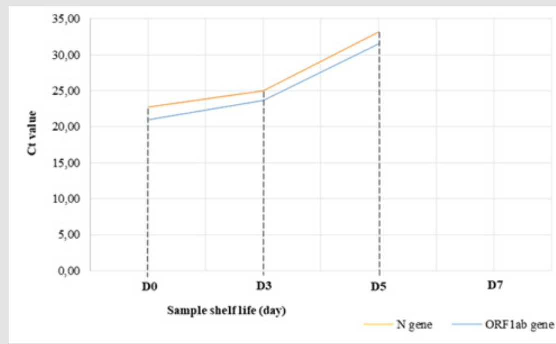

Figure 3: Comparative reverse transcription real-time polymerase chain reaction (RT-qPCR) results we obtained by comparing the shelf life (“D0”, “D3”, “D5”, and “D7”) of a total of 15 nasopharyngeal sw samples from patients known to be positive for COVID-19. The data is presented illustrativel demonstrating the difference in the cycle threshold (Ct) values in an average format between the resul obtained.

Supplementary Table 2: The cycle threshold (Ct) results for the amplification of the ORF1ab and N genes of the SARS-CoV-2 virus were analyzed in a total of 15 (fifteen) nasopharyngeal secretion samples from COVID-19 positive patients, comparing the shelf life (“D0,” “D3,” “D5,” and “D7”) of these samples.

Sample Shelf life (Day)													
		1st Day			3rd Day			5th Day			7th Day		
	Sample Number	ORF1ab gene (Ct value)	N gene (Ct value)	Result	ORF1ab gene (Ct value)	N gene (Ct value)	Result	ORF1ab gene (Ct value)	N gene (Ct value)	Result	ORF1ab gene (Ct value)	N gene (Ct value)	Result
Total number of samples: 15 (COVID-19 positive)	1	19,7	22,3	Positive*	23,8	24,3	Positive	31,3	33,6	Negative**	Not detected***	Not detected	No result****
	2	21	21,9	Positive	25,3	26,7	Positive	32,4	36,5	Negative	Not detected	Not detected	No result
	3	21,3	22,7	Positive	24,1	26	Positive	31,9	32,3	Negative	Not detected	Not detected	No result
	4	17,5	19,1	Positive	19,9	21,4	Positive	31,4	32,3	Negative	Not detected	Not detected	No result
	5	18,9	20,3	Positive	20,4	21,9	Positive	32,1	33,4	Negative	Not detected	Not detected	No result
	6	23,4	24,1	Positive	25,3	26,7	Positive	31,7	32,9	Negative	Not detected	Not detected	No result
	7	27,7	29,4	Positive	29,8	30,2	Positive	33,2	34,9	Negative	Not detected	Not detected	No result
	8	21,3	23,2	Positive	23,7	24,6	Positive	30,9	32,4	Negative	Not detected	Not detected	No result
	9	19,9	21,5	Positive	21,3	23,7	Positive	31,6	33,7	Negative	Not detected	Not detected	No result
	10	17,5	19,7	Positive	19,8	21,4	Positive	30,3	32,1	Negative	Not detected	Not detected	No result
	11	18,9	20,3	Positive	20,5	22,3	Positive	31,8	33,4	Negative	Not detected	Not detected	No result
	12	23,3	24,8	Positive	25,9	27,1	Positive	32,5	33,7	Negative	Not detected	Not detected	No result
	13	24,5	25,6	Positive	26,8	27,3	Positive	29,7	31,7	Positive	Not detected	Not detected	No result
	14	22,1	23,7	Positive	25,1	26,7	Positive	30	32,3	Negative	Not detected	Not detected	No result
	15	20,7	22,3	Positive	23,2	24,6	Positive	31,6	33,2	Negative	Not detected	Not detected	No result

Note: “Positive*” = positive for COVID-19; “Negative**” = negative for COVID-19; “Not detected***” = viral material not detected and “No result****” = no defined result.

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Table 2: Comparative reverse transcription real-time polymerase chain reaction (RT-qPCR) results were obtained by comparing the shelf life ("D0", "D3", "D5", and "D7") of a total of 15 nasopharyngeal swab samples from patients known to be positive for COVID-19. The data are presented as the mean ± standard deviation (SD) of cycle threshold (Ct) values for the ORF1ab and N genes of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), along with the p-value for the comparison of the average results obtained.

	ORF lab gene			N gene		
	Mean (Ct Value)	Standard deviation (Ct value)		Mean (Ct Value)	Standard deviation (Ct value)	
D0	21,0	2,74	p-value=0,0003615	22,73	2,63	p-value=0,0003606
		2,87			2,56	
D3	23,66		p-value=0,03052	24,99		p-value=0,03052
D5	31,49	0,95		33,23	1,23	
			p-value= Invalid			p-value= Invalid
D7	Not detected	Not detected		Not detected	Not detected	

Step 3

Both aliquots from 300 patients with clinical suspicion of COVID-19 but without a definitive diagnosis were previously frozen (~-18 ºC) for 30 minutes. The RNA extraction process for the SARS-CoV- 2 virus was performed on one aliquot, while the virus inactivation process was conducted on the other (Figure 4) (Supplementary Table 3). Of these samples, 2 (nos. 46 and 127) were excluded due to discrepancies in the results, requiring retesting for comfirmation (Supplementary Table 04). Of the remaining 298 samples, 210 tested positives for COVID-19, while 88 tested negatives. In all samples where RNA was extracted from the SARS-CoV-2 virus using the gold standard method, a delay in the amplification of the ORF1ab and N genes was observed compared to inactivation. ORF1ab gene: The average Ct values were 21.79 and 19.98, with standard deviations (SD) of 3.28 and 3.24 cycles, respectively (p-value = 2.2e-16). N gene: The average Ct values were 23.75 and 22.11, with SDs of 3.18 and 3.19 cycles, respectively (p-value = 2.2e-16) (Table 3).

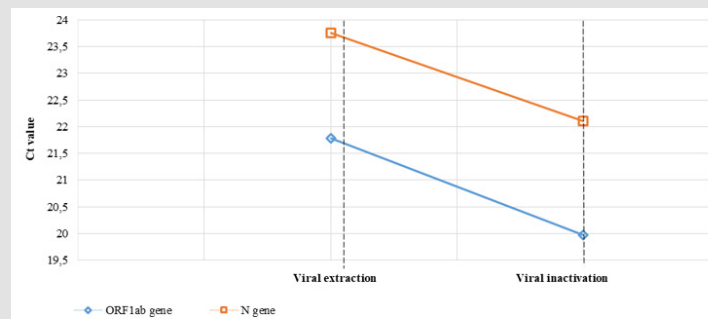

Figure 4: Comparative reverse transcription real-time polymerase chain reaction (RT-qPCR) results were obtained by comparing two methods for releasing the genetic material of the COVID-19 virus ("Viral extraction" and "Viral inactivation") from a total of 300 nasopharyngeal swab samples from patients without a definitive COVID-19 diagnosis. The data is presented illustratively to show, in an average format, the differences in cycle threshold (Ct) values between the results obtained.

Table 3: Comparative reverse transcription real-time polymerase chain reaction (RT-qPCR) results were obtained by comparing two processes for releasing the genetic material of the COVID-19 virus ("Viral extraction" and "Viral inactivation") from a total of 300 nasopharyngeal swab samples from patients without a definitive COVID-19 diagnosis. The data are presented as mean ± standard deviation (SD) of cycle threshold (Ct) values for the ORF1ab and N genes of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), along with the p-value for the comparison of the average results obtained.

	Viral extraction		Viral inactivation		
	Mean (Ct Value)	Standard deviation (Ct value)	Mean (Ct Value)	Standard deviation (Ct value)	
ORF lab gene	21,79	3,28	19,98	3,24	p-value=0,00000000000000022
N gene	23,75	3,18	22,11	3,19	p-value=0,00000000000000022

Supplementary Table 3: The cycle threshold (Ct) results for the amplification of the ORF1ab and N genes of the SARS-CoV-2 virus were analyzed in a total of 300 (three hundred) nasopharyngeal swab samples from patients without a definitive COVID-19 diagnosis, comparing two processes for releasing the genetic material of the COVID-19 virus ("Viral extraction" and "Viral inactivation") – Continued**.**

Types of processes for releasing the genetic material of the COVID-19 virus							
		Viral extraction			Viral inactivation		
	Sample Number	ORF1ab gene (Ct value)	N gene (Ct value)	Result	ORF1ab gene (Ct value)	N gene (Ct value)	Result
Total number of samples: 300 (no defined diagnosis for COVID-19)	1	17,3	19,4	Positive*	15,4	18,3	Positive
	2	18,4	19,7	Positive	16,9	18,4	Positive
	3	34,5	Not detected***	Negative**	33,9	34,1	Negative
	4	32,8	33,6	Negative	31,7	32,4	Negative
	5	33,2	34,5	Negative	32,9	33,1	Negative
	6	31,9	32,4	Negative	30,7	31,2	Negative
	7	33,7	34,1	Negative	32,8	33,4	Negative
	8	20,5	22,1	Positive	18,5	20,3	Positive
	9	19,6	21,5	Positive	18,3	19,4	Positive
	10	18,4	20,6	Positive	17,5	18,9	Positive
	11	19,3	21,7	Positive	18,4	20,3	Positive
	12	19,5	21,4	Positive	18,7	20,8	Positive
	13	17,4	19,2	Positive	15,9	17,5	Positive
	14	16,3	18,5	Positive	15,2	17,5	Positive
	15	19,3	21,5	Positive	18,1	20,4	Positive
	16	21	23,4	Positive	20,3	22,2	Positive
	17	33,4	35,1	Negative	31,3	33,1	Negative
	18	22,7	24,8	Positive	21,7	23,3	Positive
	19	24,8	25,7	Positive	22,8	23,9	Positive
	20	21,9	23,1	Positive	19,9	21,7	Positive
	21	25,4	27,2	Positive	24,6	25,9	Positive
	22	29	30,1	Positive	27,8	29,3	Positive
	23	27,4	29,4	Positive	26,4	27,7	Positive
	24	32,9	34,6	Negative	31,4	33,9	Negative
	25	33,3	34,2	Negative	31,3	32,7	Negative
	26	19,6	21,8	Positive	17,5	19,8	Positive
	27	19,2	21,4	Positive	17,9	19,3	Positive
	28	20,4	21,7	Positive	19,9	20,9	Positive
	29	21,3	23,5	Positive	19,4	22,1	Positive
	30	27,4	29,4	Positive	26,2	27,9	Positive
	31	17,8	19,8	Positive	15,9	17,2	Positive
	32	19,3	21,3	Positive	18,7	20,4	Positive
	33	18,4	21,7	Positive	16,7	20,1	Positive
	34	17,2	19	Positive	15,9	18,7	Positive
	35	19,9	21,3	Positive	18,2	20,3	Positive
	36	20,6	22,1	Positive	19,3	21,7	Positive
	37	23,5	24,8	Positive	22,3	23,4	Positive
	38	21	22,9	Positive	19,8	21,3	Positive
	39	23,9	25,4	Positive	22,1	23,7	Positive

	40	18,3	20,3	Positive	17,4	19,8	Positive
	41	32,5	34,7	Negative	30,4	32,8	Negative
	42	19,4	21,3	Positive	18,7	20,8	Positive
	43	18,2	20,4	Positive	16,9	18,7	Positive
	44	20,5	22,5	Positive	19,1	21,3	Positive
	45	21,7	23,8	Positive	19,8	22,4	Positive
	46	29,7	30,3	Positive	31,2	32,9	Negative
	47	22,9	24,3	Positive	21,4	23,2	Positive
	48	23,5	25,7	Positive	22,1	24,2	Positive
	49	23,3	25,9	Positive	21,9	24,3	Positive
	50	29,4	31	Positive	27,6	29,9	Positive
	51	26,8	27,7	Positive	25,4	26,4	Positive
	52	23,9	25,2	Positive	22,1	24,3	Positive
	53	21,4	23,4	Positive	19,8	21,7	Positive
	54	33,3	34,9	Negative	31,7	31,9	Negative
	55	23,4	25,7	Positive	21,3	23,4	Positive
	56	34,7	35,1	Negative	32,3	32,9	Negative
	57	33	34,6	Negative	31,3	32,1	Negative
	58	32,3	34,4	Negative	30,6	32,7	Negative
	59	33,5	34,7	Negative	31,7	32,1	Negative
	60	29,3	30,3	Positive	27,9	29,4	Positive
	61	27,2	29,3	Positive	25,3	27,6	Positive
	62	28,3	30,2	Positive	26,7	29,1	Positive
	63	21,9	23,5	Positive	19,4	22,3	Positive
	64	16,3	19,3	Positive	15,9	17,2	Positive
	65	19,4	21,3	Positive	17,5	19,8	Positive
	66	19,7	21,7	Positive	17,4	19,3	Positive
	67	21,3	23,5	Positive	18,9	22,3	Positive
	68	34,2	35,3	Negative	32,7	33,2	Negative
	69	33,4	34,1	Negative	31,9	32,6	Negative
	70	20,4	22,3	Positive	18,7	21,3	Positive
	71	21,3	23,6	Positive	19,6	21,1	Positive
	72	22,6	24,3	Positive	20,9	22,3	Positive
	73	22,3	24,1	Positive	20,7	21,9	Positive
	74	22,9	24,7	Positive	20,9	22,7	Positive
	75	20,1	22,3	Positive	18,6	21,3	Positive
	76	25,3	27,4	Positive	23,7	25,8	Positive
	77	21,8	23,5	Positive	19,3	21,7	Positive
	78	20,3	21,9	Positive	18,1	19,9	Positive
	79	19,4	21,6	Positive	17,5	20,3	Positive
	80	20,4	22,3	Positive	18,9	21,2	Positive
	81	20,6	22,6	Positive	19,3	21,5	Positive
	82	19,3	20,9	Positive	17,6	19,4	Positive
	83	18,3	20,6	Positive	16,9	18,1	Positive
	84	19,2	21,5	Positive	17,8	19,8	Positive

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	85	32,4	33,6	Negative	30,3	31,4	Negative
	86	33,1	34,6	Negative	31,1	32,3	Negative
	87	32,5	33,3	Negative	30,9	31,3	Negative
	88	17,3	19,5	Positive	15,4	17,5	Positive
	89	20,4	22,3	Positive	18,7	21,3	Positive
	90	21,9	23,7	Positive	19,6	21,3	Positive
	91	21,1	23,5	Positive	19,3	21,6	Positive
	92	20,6	22,1	Positive	18,7	20,9	Positive
	93	19,8	21,8	Positive	18,4	19,7	Positive
	94	19,4	21,3	Positive	18,1	19,4	Positive
	95	33,9	34,5	Negative	32,7	33,7	Negative
	96	32,1	33,9	Negative	31	32,8	Negative
	97	31,6	32,7	Negative	31,4	31,2	Negative
	98	32,7	33,8	Negative	31,9	32,6	Negative
	99	32,7	33,4	Negative	31,5	32,1	Negative
	100	17,4	19,9	Positive	15,6	17,2	Positive
	101	19,3	21,7	Positive	17,3	19,9	Positive
	102	19,2	21,5	Positive	17,8	19,4	Positive
	103	20,4	22,3	Positive	18,7	20,7	Positive
	104	20	21,8	Positive	17,7	19,9	Positive
	105	21,4	22,6	Positive	19,4	21,3	Positive
	106	19,8	21,3	Positive	17,8	19,8	Positive
	107	19,3	20,9	Positive	17,2	19,5	Positive
	108	20,4	23,2	Positive	19,8	22,3	Positive
	109	23,1	25,3	Positive	21,1	23,9	Positive
	110	27,2	29,1	Positive	25,6	27,1	Positive
	111	17,9	19,7	Positive	15,9	17	Positive
	112	32,3	33,1	Negative	30,4	31,9	Negative
	113	33,8	34,9	Negative	32,4	33,1	Negative
	114	31,6	32,4	Negative	31,1	32	Negative
	115	18,3	20,4	Positive	16,9	19,1	Positive
	116	20,3	22,8	Positive	18,2	20,5	Positive
	117	21,8	23,5	Positive	19,7	21,2	Positive
	118	19,3	21,4	Positive	17,4	19,9	Positive
	119	20,9	22,8	Positive	18,3	21,3	Positive
	120	20,1	22,1	Positive	18,5	21,3	Positive
	121	21,3	23,6	Positive	19,3	21,9	Positive
	122	24,3	26,7	Positive	22	24,7	Positive
	123	25,9	27,4	Positive	22,9	24,5	Positive
	124	22,8	24,9	Positive	19,3	21,8	Positive
	125	31,9	33,3	Negative	30,7	32,1	Negative
	126	21,4	23,5	Positive	19,4	22,3	Positive
	127	29,3	30,6	Positive	31,7	32,4	Negative
	128	27,4	29,1	Positive	25,1	27,3	Positive
	129	33,9	34,4	Negative	31,9	32,6	Negative
	130	28,3	29,6	Positive	26,7	28,3	Positive

	131	19,1	20,1	Positive	17,3	19,9	Positive
	132	27	29,4	Positive	25,2	27,9	Positive
	133	23,1	25,6	Positive	20,3	22,8	Positive
	134	32,5	33,1	Negative	32,1	32,7	Negative
	135	19,4	21,3	Positive	17,5	19,8	Positive
	136	23,2	25,7	Positive	21,3	24,9	Positive
	137	25,7	27,4	Positive	23,1	25,6	Positive
	138	20,1	22,3	Positive	18,7	20,3	Positive
	139	19,3	21,9	Positive	17,5	19,6	Positive
	140	22,4	24,7	Positive	21,2	23,7	Positive
	141	25,7	27,5	Positive	23,5	25,4	Positive
	142	23,2	25,8	Positive	21,9	23,9	Positive
	143	22,1	24,3	Positive	19,3	21,3	Positive
	144	29,4	30,3	Positive	27,6	29,5	Positive
	145	28,7	29,5	Positive	26,3	28,1	Positive
	146	29,1	30,6	Positive	27,1	29,4	Positive
	147	33,7	34,8	Negative	33,1	33,9	Negative
	148	31,7	33,6	Negative	30,9	32,3	Negative
	149	19,3	21,3	Positive	17,3	20,1	Positive
	150	17,3	19,8	Positive	15,6	17,9	Positive
	151	19,3	21,7	Positive	17,3	19,9	Positive
	152	20,3	22,3	Positive	18,1	20,3	Positive
	153	34,6	Not detected	Negative	32,3	34,1	Negative
	154	29,3	31,4	Positive	27,6	29,7	Positive
	155	19,4	21,7	Positive	17,9	19,8	Positive
	156	32,8	33,6	Negative	31,5	32,9	Negative
	157	33,8	34,7	Negative	32,6	33,2	Negative
	158	32,9	34,1	Negative	32,6	33,2	Negative
	159	31,9	32,5	Negative	30,9	31,7	Negative
	160	32,6	34,1	Negative	31,4	32,8	Negative
	161	33,2	34,5	Negative	32	33,4	Negative
	162	31,7	32,9	Negative	31,2	32,4	Negative
	163	20,4	23,1	Positive	18,3	21,1	Positive
	164	21,5	22,7	Positive	19,2	21,5	Positive
	165	20,4	21,9	Positive	18,4	20,3	Positive
	166	32,4	33,6	Negative	31,7	32,5	Negative
	167	34,7	34,9	Negative	32,9	33,4	Negative
	168	33,7	34,3	Negative	32,8	33,5	Negative
	169	23,8	25,7	Positive	21,6	23,9	Positive
	170	23,4	25,3	Positive	21,2	23,6	Positive
	171	32,2	34,1	Negative	31,7	32,8	Negative
	172	31,9	33,6	Negative	30,6	32,1	Negative
	173	19,8	21,7	Positive	17,6	19,8	Positive
	174	19,3	21,4	Positive	17,9	21,1	Positive
	175	21,4	23,4	Positive	19,8	21,9	Positive
	176	33,4	34,4	Negative	32,9	33,2	Negative

	177	23,5	25,6	Positive	21,7	23,4	Positive
	178	22,9	24,9	Positive	20,3	22,5	Positive
	179	23,1	25,7	Positive	21,8	23,3	Positive
	180	24,5	26,3	Positive	22,9	24,6	Positive
	181	23,8	25,7	Positive	21,7	23,1	Positive
	182	23,1	25,3	Positive	20,8	23,8	Positive
	183	24,7	26,2	Positive	22,1	24,9	Positive
	184	19,8	21,8	Positive	17,4	19,7	Positive
	185	19,2	21,1	Positive	17,1	19,8	Positive
	186	18,4	20,1	Positive	15,9	18,3	Positive
	187	17,3	19,8	Positive	15,4	18,1	Positive
	188	19,3	21,4	Positive	17,5	19,5	Positive
	189	20,4	22,6	Positive	18,3	20,1	Positive
	190	32,1	33,3	Negative	31,1	31,9	Negative
	191	20,1	23,1	Positive	18,7	19,3	Positive
	192	22,3	24,5	Positive	21,3	23,4	Positive
	193	23,7	25,6	Positive	21,8	24,8	Positive
	194	23,3	26,9	Positive	22,1	25,4	Positive
	195	32,9	33,7	Negative	31,7	32,5	Negative
	196	24,3	26,7	Positive	22,5	24,1	Positive
	197	19,8	21,5	Positive	17,4	19,3	Positive
	198	17,6	19,8	Positive	15,3	17,4	Positive
	199	17,9	20,1	Positive	15,9	17,6	Positive
	200	18,4	20,2	Positive	16,3	18,9	Positive
	201	18,2	20,3	Positive	16,1	18,5	Positive
	202	19,3	17,9	Positive	18,7	20,8	Positive
	203	20,4	22,7	Positive	19,8	21,4	Positive
	204	33,8	34,3	Negative	32,1	33,7	Negative
	205	17,8	18,9	Positive	15,7	19,3	Positive
	206	19,3	21,6	Positive	17,3	20,3	Positive
	207	20,3	22,1	Positive	18,4	20,4	Positive
	208	21,8	23,4	Positive	19,2	21,3	Positive
	209	27,5	29,3	Positive	25,9	27,1	Positive
	210	28,4	30,2	Positive	27,3	29,8	Positive
	211	17,4	19,8	Positive	15,7	18,5	Positive
	212	24,9	26,3	Positive	22,1	24,2	Positive
	213	33,2	34,5	Negative	32,7	33,3	Negative
	214	34,8	Not detected	Negative	33,9	Not detected	Negative
	215	31,5	32,8	Negative	30,7	31,6	Negative
	216	32,9	33,6	Negative	31,4	32,1	Negative
	217	33,9	34,5	Negative	32,8	33,9	Negative
	218	34,4	Not detected	Negative	33,3	Not detected	Negative
	219	33,1	34,6	Negative	32,4	33,1	Negative
	220	25,8	27,3	Positive	23,2	25,6	Positive

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	221	27,3	29,7	Positive	25,8	27,6	Positive
	222	29,3	31,3	Positive	27,1	29,7	Positive
	223	19,4	21,3	Positive	17,3	19,5	Positive
	224	27,4	29,5	Positive	25,4	27,3	Positive
	225	17	19,8	Positive	15,2	17,8	Positive
	226	28,3	29,7	Positive	26,5	28,3	Positive
	227	23,5	25,6	Positive	21,3	23,4	Positive
	228	33,1	33,8	Negative	31,2	32,9	Negative
	229	34,5	34,9	Negative	32,1	33,7	Negative
	230	29,3	30,3	Positive	27,3	29,7	Positive
	231	27,7	29,3	Positive	25,7	27,3	Positive
	232	23,9	25,1	Positive	21,3	23,4	Positive
	233	24,7	27,5	Positive	22,1	24,8	Positive
	234	23,1	25,6	Positive	21,9	23,5	Positive
	235	19,4	21,8	Positive	17,3	19,8	Positive
	236	18,7	20,3	Positive	16,7	18,4	Positive
	237	33,2	34,2	Negative	32,6	33,1	Negative
	238	34,1	Not detected	Negative	33,5	34,3	Negative
	239	34,7	Not detected	Negative	33,7	34,5	Negative
	240	32,5	33,6	Negative	31,2	32,8	Negative
	241	33,1	34,2	Negative	32,6	33,4	Negative
	242	31,1	32,5	Negative	30,8	31,5	Negative
	243	19,3	22,3	Positive	17,6	20,2	Positive
	244	19,7	22,8	Positive	17,5	19,8	Positive
	245	21,5	24,1	Positive	19,6	21,4	Positive
	246	20,8	22,1	Positive	18,9	20,5	Positive
	247	20,9	22,3	Positive	19,3	21,9	Positive
	248	18,3	20,4	Positive	16,9	18,9	Positive
	249	19,2	21,3	Positive	17,8	19,9	Positive
	250	19,4	21,3	Positive	18,3	20,7	Positive
	251	20,5	22,6	Positive	18,7	20,4	Positive
	252	33,8	34,7	Negative	32,1	33,4	Negative
	253	34,6	Not detected	Negative	33,9	34,7	Negative
	254	31,9	32,6	Negative	30,8	31,4	Negative
	255	32,5	33,6	Negative	30,9	31,8	Negative
	256	33,4	34,2	Negative	32,7	33,2	Negative
	257	31,9	32,1	Negative	30,6	31,3	Negative
	258	32,7	33,2	Negative	31,9	32,7	Negative
	259	34,8	Not detected	Negative	33	34,6	Negative
	260	33,5	34,3	Negative	32,4	33,1	Negative
	261	32,9	34,1	Negative	31,7	32,9	Negative
	262	21,6	23,7	Positive	18,7	21,3	Positive
	263	22,9	24,5	Positive	19,9	21,7	Positive
	264	25,4	27,6	Positive	23,4	25,9	Positive
	265	24,9	26,8	Positive	22,8	24,6	Positive
	266	18,4	20,3	Positive	16,5	18,1	Positive

	267	32,6	33,4	Negative	31,7	32,8	Negative
	268	32,9	33,2	Negative	31,1	32,3	Negative
	269	24,3	26,9	Positive	22,1	24,5	Positive
	270	31,6	32,4	Negative	30,3	31,9	Negative
	271	29,4	31,4	Positive	27,3	29,4	Positive
	272	22,1	24,3	Positive	20,2	22,3	Positive
	273	21,7	23,7	Positive	19,8	22,1	Positive
	274	29,8	30,3	Positive	27,6	29,9	Positive
	275	33,7	34,5	Negative	32,5	33,8	Negative
	276	32,7	33,2	Negative	30,9	31,7	Negative
	277	19,6	21,7	Positive	18,4	20,3	Positive
	278	18,4	20,3	Positive	16,9	19,1	Positive
	279	20,3	22,4	Positive	18,7	20,3	Positive
	280	21,7	23,5	Positive	19,3	21,5	Positive
	281	33,6	34,3	Negative	32,7	33,5	Negative
	282	31,1	32,7	Negative	30,9	31,4	Negative
	283	20,8	22,3	Positive	18,4	21,3	Positive
	284	21,4	24,2	Positive	19,3	22,4	Positive
	285	22,9	24,8	Positive	20,6	22,8	Positive
	286	23,1	25,6	Positive	21,2	23,4	Positive
	287	28,3	30,1	Positive	26,7	28,6	Positive
	288	33,9	34,5	Negative	32,5	33,3	Negative
	289	32,6	33,7	Negative	30,8	32,1	Negative
	290	27,4	29,1	Positive	24,9	26,9	Positive
	291	17,5	19,5	Positive	15,6	17,5	Positive
	292	19,8	22,3	Positive	17,4	20,2	Positive
	293	20,5	23,2	Positive	18,3	22,4	Positive
	294	19,4	21,6	Positive	17,6	19,7	Positive
	295	33,9	34,8	Negative	32,5	33,1	Negative
	296	34,6	Not detected	Negative	33,5	34,2	Negative
	297	18,7	21,3	Positive	17,5	19,8	Positive
	298	32,5	33,7	Negative	30,7	32,4	Negative
	299	33,5	34,3	Negative	32,7	33,5	Negative
	300	33,4	Not detected	Negative	31,3	33,1	Negative

Note: “Positive*” = positive for COVID-19; “Negative**” = negative for COVID-19 and “Not detected***” = viral material not detected.

Supplementary Table 4: The cycle threshold (Ct) results for the amplification of the ORF1ab and N genes of the SARS-CoV-2 virus from the re-test of 02 (two) nasopharyngeal swab samples (number 46 and 127) from patients with discrepancies in results when comparing the two processes for releasing the genetic material of the COVID-19 virus ("Viral extraction" and "Viral inactivation"). The re-test was conducted using the QIAGEN kit ("QIA amp Viral RNA Mini Kit").

		Types of processes for releasing the genetic material of the COVID-19 virus (COUNTERTEST)			Types of processes for releasing the genetic material of the COVID-19 virus (RETEST)		
		Viral extraction (QIA amp Viral RNA Mini Kit)			Viral inactivation		
	Sample Number	ORF lab gene (Ct value)	N gene (Ct value)	Result	ORF lab gene (Ct value)	N gene (Ct value)	Result
Total number of samples: 02 (samples with diagnosis for COVID-19 to be confirmed)	46	28,9	30,3	Positive	27,6	29,4	Positive
	127	29,1	30,8	Positive	21,3	30,1	Positive

Note: Legend: "Positive*" = positive for COVID-19.

Step 4

Four aliquots were taken from 15 patients known to be positive for COVID-19, with each aliquot being previously frozen (~-18 ºC) before undergoing the SARS-CoV-2 virus inactivation process. The difference between these aliquots was the volume of "Proteinase K" added: 25 μL to the 1st aliquot, 15 μL to the 2nd, 10 μL to the 3rd, and 5 μL to the 4th (Figure 5) (Supplementary Table 5). An increase in the amplification delay of the ORF1ab and N genes was observed as the enzyme volume decreased. For the ORF1ab gene, the average Ct values were 19.91, 26.80, 33.79, and Not Detected, with standard deviation (SD) values of 1.93, 2.30, 2.13, and invalid cycles, respectively. Comparing the addition of 25 μL to 15 μL yielded a statistically significant p-value of 0.0007, while comparing 15 μL to 10 μL resulted in a p-value of 3.052e-05. For the N gene, the average Ct values were 21.47, 34.14, 37.21, and Not Detected, with SD values of 1.88, 2.09, 2.97, and invalid cycles, respectively. Comparing the addition of 25 μL to 15 μL yielded a p-value of 3.052e-05, and comparing 15 μL to 10 μL resulted in a p-value of 0.004181 (Table 4).

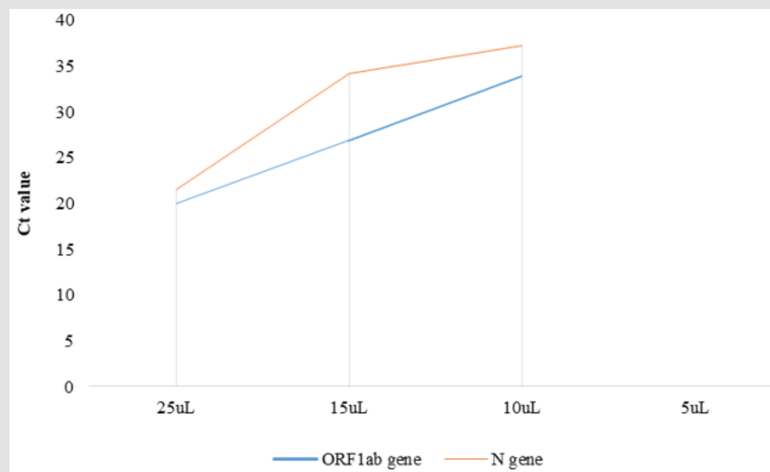

Figure 5: Comparative reverse transcription real-time polymerase chain reaction (RT-qPCR) results were obtained to compare the amounts of proteinase K added to the reaction—25 μL, 15 μL, 10 μL, and 5 μL—using a total of 15 nasopharyngeal swab samples from patients known to be positive for COVID-19. The data are presented illustratively to demonstrate the average differences in cycle threshold (Ct) values among the results obtained.

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Supplementary Table 5: The cycle threshold (Ct) results for the amplification of the ORF1ab and N genes of the SARS-CoV-2 virus were analyzed in a total of 15 (fifteen) nasopharyngeal swab samples from patients known to be positive for COVID-19, comparing the amounts of proteinase K added to the reaction—25 μL, 15 μL, 10 μL, and 5 μL.

Amount of Proteinase K added to the reaction (μL)													
		25μL				15μL		10μL				5μL	
	Sample Number	ORF1ab gene (Ct value)	N gene (Ct value)	Result	ORF1ab gene (Ct value)	N gene (Ct value)	Result	ORF1ab gene (Ct value)	N gene (Ct value)	Result	ORF1ab gene (Ct value)	N gene (Ct value)	Result
Total number of samples: 15 (COVID-19 positive)	1	20,3	22,4	Positive	29,4	33,6	Positive*	34,6	38,3	Negative	Not detected***	Not detected	No result****
	2	21,3	23,2	Positive	30,2	35,9	Negative**	32,4	36,7	Negative	Not detected	Not detected	No result
	3	19,5	21,3	Positive	29,5	33,9	Positive	34,1	39,1	Negative	Not detected	Not detected	No result
	4	17,3	19,4	Positive	23,5	29,9	Positive	27,9	31,2	Positive	Not detected	Not detected	No result
	5	22,4	24,3	Positive	26,8	34,1	Positive	33,7	38,3	Negative	Not detected	Not detected	No result
	6	18,5	19,8	Positive	23	30,2	Positive	31,3	35,4	Negative	Not detected	Not detected	No result
	7	20,3	22,1	Positive	28,4	32,7	Positive	34,5	39,4	Negative	Not detected	Not detected	No result
	8	21,4	22,5	Positive	25	33,4	Positive	33,6	37,9	Negative	Not detected	Not detected	No result
	9	22,9	24,1	Positive	26,9	34,8	Positive	33,9	37,6	Negative	Not detected	Not detected	No result
	10	17,3	19,3	Positive	25,4	33,7	Positive	35,1	39,4	Negative	Not detected	Not detected	No result
	11	18,5	19,5	Positive	26,3	35,2	Positive	34,3	39,2	Negative	Not detected	Not detected	No result
	12	17	18,7	Positive	25,2	36,9	Positive	35,8	38,2	Negative	Not detected	Not detected	No result
	13	19,3	20,3	Positive	25,4	36,7	Positive	36,1	39,5	Negative	Not detected	Not detected	No result
	14	20,4	21,5	Positive	26,9	34,7	Positive	36,5	38,3	Negative	Not detected	Not detected	No result
	15	22,3	23,6	Positive	30,1	36,4	Negative	33,1	29,7	Negative	Not detected	Not detected	No result

Note: "Positive*" = positive for COVID-19; "Negative**" = negative for COVID-19; "Not detected***" = viral material not detected and "No result****" = no defined result.

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Table 4: Comparative reverse transcription real-time polymerase chain reaction (RT-qPCR) results were analyzed to compare the amounts of proteinase K added to the reaction in µL ("25 µL," "15 µL," "10 µL," and "5 µL") across a total of 15 nasopharyngeal swab samples from patients known to be positive for COVID-19. The data are presented as the mean ± standard deviation (SD) of cycle threshold (Ct) values for the ORF1ab and N genes of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), along with the p-values comparing the average results obtained.

ORF lab gene				N gene		
	Mean (Ct Value)	**Standard deviation (Ct value)**		**Mean (Ct Value)**	**Standard deviation (Ct value)**	
25µL	19,91	1,93	p-value=0,0007	21,47	1,88	p-value=0,0003052
		2,30			2,09	
15µL	26,80		p-value=0,03052	34,14		p-value=0.004181
		2,13			2,97	
10µL	33,79			37,21		
			p-value= Invalid			p-value= Invalid
5µL	Not detected	Not detected		Not detected	Not detected	

Step 5

Two aliquots were taken from 30 patients known to be positive for COVID-19, with each aliquot previously frozen (~-18 ºC) before undergoing the SARS-CoV-2 virus inactivation process. The difference between these aliquots was in the equipment used: the first aliquot was processed using the Dry Water Bath NT 362 – DryBlo, while the second was processed with the thermoblock equipped with an agitator (MTC- 100 Thermo Shaker Incubator - Hangzhou Mil Instruments Co. Ltda) (Figure 6) (Supplementary Table 5). An earlier amplification of the ORF1ab and N genes was observed in the second aliquot. ORF1ab gene: The average Ct values were 21.70 and 19.61, with standard deviations (SD) of 2.43 and 1.93 cycles, respectively (p-value = 1.342e-06). N gene: The average Ct values were 23.77 and 21.32, with SD values of 2.32 and 1.82 cycles, respectively (p-value = 1.329e-06) (Table 5) (Supplementary Table 6).

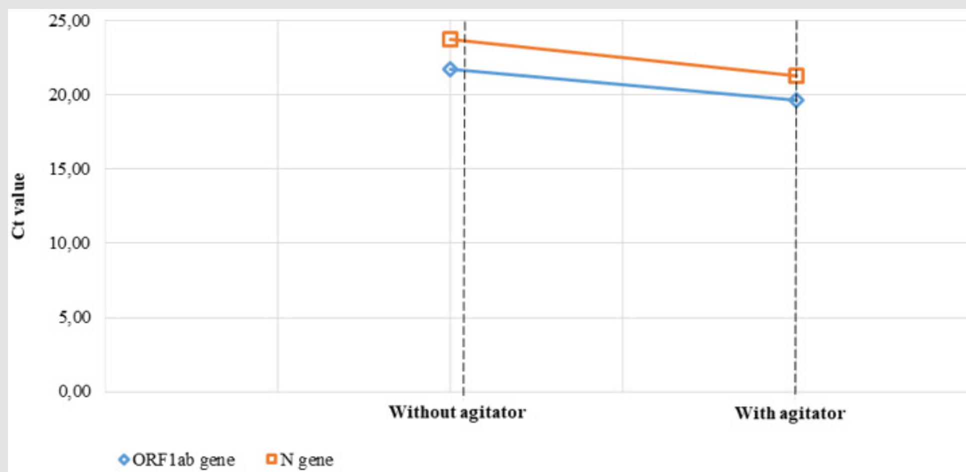

Figure 6: Comparative reverse transcription real-time polymerase chain reaction (RT-qPCR) results were obtained by comparing the viral RNA incubation step at elevated temperature ("Without agitator" and "With agitator") from a total of 30 nasopharyngeal swab samples from patients known to be positive for COVID-19. The data is presented in an illustrative manner to highlight, in an average format, the differences in cycle threshold (Ct) values between the results obtained.

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Table 5: Comparative reverse transcription real-time polymerase chain reaction (RT-qPCR) results comparing viral RNA incubation steps at elevated temperatures ("Without agitator" and "With agitator") from a total of 30 nasopharyngeal swab samples of patients known to be positive for COVID-19. The data are presented as mean ± standard deviation (SD) of cycle threshold (Ct) values for the ORF1ab and N genes of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), along with the p-value from the comparison of the average results.

Without agitator			With agitator		
	Mean (Ct Value)	Standard deviation (Ct value)	Mean (Ct Value)	Standard deviation (Ct value)	
ORF lab gene	21,70	2,43	19,61	1,93	p-value=0,001342
N gene	23,77	2,32	21,32	1,82	p-value=0,001329

Supplementary Table 6: The cycle threshold (Ct) results for the amplification of the ORF1ab and N genes of the SARS-CoV-2 virus were analyzed in a total of 30 (thirty) nasopharyngeal swab samples from patients known to be positive for COVID-19, comparing the viral RNA incubation step at elevated temperature ("Without agitator" and "With agitator").

Viral RNA incubation step at elevated temperature							
		Without agitator			With agitator		
	Sample Number	ORF1ab gene (Ct value)	N gene (Ct value)	Result	ORF1ab gene (Ct value)	N gene (Ct value)	Result
Total number of samples: 30 (COVID-19 positive)	1	23,8	25,8	Positive*	21,3	23,2	Positive
	2	22,7	24,7	Positive	21,6	22,6	Positive
	3	24,6	26,3	Positive	22,4	24,2	Positive
	4	21,3	24,2	Positive	19,4	21,7	Positive
	5	19,4	22,1	Positive	17,3	19,8	Positive
	6	19,7	22,7	Positive	17,8	20,2	Positive
	7	22,1	23,6	Positive	20,2	22,6	Positive
	8	22,6	23,4	Positive	21,4	23,4	Positive
	9	21	23,1	Positive	19,7	21,7	Positive
	10	21,1	23,7	Positive	20,1	21,9	Positive
	11	19,8	22,1	Positive	18,4	20,3	Positive
	12	17,6	20,3	Positive	15,4	17,9	Positive
	13	20,9	22,5	Positive	19,7	21,4	Positive
	14	21,4	23,9	Positive	21,4	22,3	Positive
	15	23,1	25,4	Positive	20,5	22,7	Positive
	16	23,5	25,9	Positive	20,4	22,3	Positive
	17	24,3	26,3	Positive	22,7	23,6	Positive
	18	19,3	21,3	Positive	17,4	19,3	Positive
	19	24,3	26,2	Positive	22,7	23,7	Positive
	20	19,5	21,7	Positive	17,7	19,1	Positive
	21	20,4	22	Positive	18,5	20,3	Positive
	22	21,5	23,4	Positive	19,4	20,8	Positive
	23	22,7	24,3	Positive	21,2	22,5	Positive
	24	19,6	21	Positive	17,3	18,6	Positive
	25	20,4	22,1	Positive	19,3	20,3	Positive
	26	24,1	26,4	Positive	22,3	23,9	Positive
	27	22,8	24,9	Positive	20,1	22,3	Positive
	28	18,4	21	Positive	17,2	18,8	Positive
	29	29,7	31,4	Positive	18,3	19,6	Positive
	30	19,4	21,5	Positive	17,3	18,5	Positive

Note: "Positive*" = positive for COVID-19.

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Statistical Analyses

The Ct values obtained in real-time RT-PCR reactions performed in all tests were compared by tests and p values < 0.05 were considered significant differences [48-53].

Discussion

COVID-19

During the COVID-19 pandemic, there was an increased demand for RT-PCR testing, making it crucial to develop rapid and effective methods for the extraction and inactivation of SARS-CoV- 2 genetic material. These advancements aimed to facilitate analysis and reduce processing time. This surge presented significant challenges, particularly regarding response times for results and the extraction of viral genetic material [54]. As the pandemic intensified, many laboratories found themselves overwhelmed by the volume of testing requests. This situation was exacerbated by shortages in resources, personnel, and expertise, which hindered the efficient processing of samples. Although RT-PCR tests are highly accurate, they are time-consuming due to the complexity of extraction and amplification processes. Additionally, the lack of standardized procedures for sample handling and test execution contributed to variability in result quality and processing speed [55-58]. A critical issue was the RNA extraction from patient samples, a prerequisite for RT-PCR testing [54]. RNA extraction is a complex process requiring specialized equipment and trained personnel. Many laboratories faced difficulties due to the limited supply of commercial RNA extraction kits, which were in high global demand. Some research groups developed alternative protocols, while others turned to automated systems to alleviate this pressure, although these systems also required significant training and adaptation [59].

Among these research groups, we developed an alternative protocol for SARS-CoV-2 RNA extraction based on scientific evidence. This protocol demonstrated significant benefits by utilizing the prior freezing of nasopharyngeal samples containing SARS-CoV-2, combined with the use of proteinase K, elevated temperature, and agitation. This approach has been proven efficient and validated to facilitate viral RNA extraction and optimize virus detection by RT-PCR [60,61].

Prior Freezing of Nasopharyngeal Samples

The prior freezing of nasopharyngeal samples has proven to be a widely adopted practice for preserving RNA integrity and improving its extraction efficiency, particularly in RT-PCR tests for detecting SARS-CoV-2. This approach is crucial to ensuring the quality of molecular analyses, especially in scenarios where samples need to be transported or stored before processing. Studies indicate that freezing at low temperatures, such as -18 °C, -80 °C, or even lower, helps preserve the integrity of viral RNA by protecting it from enzymatic degradation, such as the action of RNases. Gao, et al. (2020) observed that prior freezing significantly contributes to the recovery of high-quality RNA, resulting in more efficient amplification during RT-PCR, even after prolonged storage periods [60]. Tariq, et al. (2020) supported these findings, demonstrating that freezing nasopharyngeal samples before RNA extraction reduces genetic material degradation and improves the quantity of RNA recovered [61]. This occurs because freezing facilitates cell rupture and the release of viral RNA without compromising the integrity of its genomic sequence. Additionally, freezing offers logistical advantages, allowing for the preservation of samples over extended periods and ensuring their stability during transportation to laboratories. Scheller, et al. (2020) highlighted that frozen samples exhibit greater stability for RNA extraction compared to fresh samples, which are more susceptible to enzymatic degradation [62,63].

Similarly, Dimeski (2020) reported that freezing is particularly advantageous for samples collected in remote locations, facilitating transport without compromising RNA quality [64,65]. The practice of freezing not only ensures the integrity of viral RNA but also improves the reliability and consistency of laboratory results. Moreover, it is essential in high-demand situations or adverse conditions, such as prolonged transportation needs, where the risk of degradation is elevated. Thus, this technique is recommended as an additional step to optimize the quality of molecular analyses and ensure the accuracy of diagnostic tests.

Elevation of the Temperature of Nasopharyngeal Samples

Raising the temperature has proven to be a crucial strategy for optimizing viral RNA extraction by facilitating the disruption of cell membranes and denaturing proteins that might interfere with the process. Studies show that heating samples, particularly to temperatures of 95 °C, effectively induces cell lysis and releases viral RNA, thereby improving the sensitivity and efficiency of diagnostic methods such as RT-PCR [25,66]. Duan, et al. (2020) demonstrated that applying heat during extraction significantly enhanced the efficiency of viral RNA release while also reducing the time required for sample preparation, resulting in greater sensitivity in molecular tests [67]. Additionally, the elevation of temperature, when combined with physical methods such as agitation, plays an essential role in breaking cell membranes, making viral RNA more accessible for subsequent analyses. This process is fundamental to the effectiveness of molecular diagnostics, especially in nasopharyngeal samples, which have a complex matrix of cells, mucosal components, and proteins. The combination of heating with the use of lytic agents, such as detergents or proteinase K, further enhances the release of viral RNA. Kozak, et al. (2020) highlighted that increasing the temperature during extraction promotes more efficient viral RNA release by breaking down cellular and viral protein structures, particularly in nasopharyngeal samples. This approach is widely supported by studies emphasizing the importance of efficient lysis for detecting viruses such as SARS-CoV-2 [68].

Addition of Proteinase K

Proteinase K played a fundamental role in enhancing laboratory protocols during the COVID-19 pandemic, particularly in the inactivation of SARS-CoV-2 and the extraction of viral RNA. This proteolytic enzyme, renowned for its robustness and efficiency under high-temperature conditions, has proven to be an indispensable tool for preserving RNA integrity in molecular analyses such as RT-PCR, which is essential for rapid and reliable virus diagnosis. Studies highlight that proteinase K maintains its activity at elevated temperatures, ranging from 56 °C to 95 °C, enabling efficient degradation of viral proteins and inactivation of the virus. This ensures the safety of professionals and the quality of the extracted RNA. Wu, et al. (2020) demonstrated that this approach results in high-quality viral RNA that is compatible with subsequent analyses, increasing efficiency in high- demand scenarios [69,70]. Haugen, et al. (2007) emphasized the stability of this enzyme at high temperatures, facilitating the efficient degradation of proteins and the release of viral RNA in clinical samples [71]. Additionally, Liu, et al. (2019) confirmed that applying proteinase K at 95 °C eliminates contaminating proteins, such as RNases, thereby improving the quality of the extracted RNA without compromising its integrity [72]. The versatility of proteinase K also extends to its application across various types of clinical samples. Verstrepen, et al. (2020) demonstrated that its use in saliva samples ensured safe virus inactivation and efficient RNA extraction [73].

Meanwhile, Zong, et al. (2020) reinforced its efficacy in nasopharyngeal samples, even under high-demand conditions such as pandemic peaks [74,75]. Moreover, combining proteinase K with heating and agitation has proven advantageous in accelerating RNA extraction and reducing the time required for molecular testing. Martins, et al. (2021) reported that this combination of heating and enzyme increased the amount of RNA extracted, while Gao, et al. (2020) highlighted that heating samples accelerates cell lysis and minimizes the impact of interfering proteins [76,77].

Use of a Shaker

Mechanical agitation during viral RNA extraction has proven to be an efficient technique for homogenizing samples and physically disrupting cells, promoting more effective RNA release. This step is essential for enhancing the sensitivity and accuracy of RT-PCR tests, ensuring reliable and consistent results. Martins et al. (2021) demonstrated that using mechanical shakers during viral RNA extraction significantly improved cell disruption, enabling more efficient sample homogenization and more precise amplification in RT-PCR [78]. Agitation accelerates RNA release by increasing the interaction between the lysis reagent and the cells or viruses present, particularly in samples with high viscosity or protein content. Zhao, et al. (2020) supported these findings, showing that the use of shakers in nasopharyngeal samples resulted in a more uniform extracted solution, which enhanced the sensitivity and accuracy of the detection process [79]. Additionally, Chen, et al. (2018) observed that mechanical agitation during extraction improved both the quantity and quality of RNA extracted from clinical samples, contributing to more precise diagnoses [80]. The efficiency of this method is also reflected in the reproducibility of results. Jiang et al. (2021) indicated that the use of shakers reduces variability among samples, ensuring consistent RNA concentrations, even when analyzing large volumes of heterogeneous samples [81]. Moreover, the proper exposure of viral RNA following cell membrane disruption, facilitated by agitation, is crucial for the sensitive detection of SARS-CoV-2. Liu et al. (2020) observed that efficient RNA exposure in nasopharyngeal samples improved amplification during RT-PCR, increasing the test's sensitivity [82].

Conclusion

The combination of prior freezing of nasopharyngeal samples, the use of proteinase K, elevated temperatures, and mechanical agitation constitutes a highly efficient and validated approach to optimizing viral RNA extraction and SARS-CoV-2 detection via RT-PCR. Freezing preserves RNA integrity by minimizing enzymatic degradation and ensures sample stability during transport and storage. Proteinase K provides a practical and reliable solution by accelerating cell lysis and protein degradation, particularly at elevated temperatures, such as 95 °C, owing to its thermostable robustness. Heating and agitation further enhance cell membrane disruption, maximizing viral RNA release and improving the sensitivity and accuracy of molecular diagnostics. These practices not only ensure high- quality RNA extraction but also reduce processing time, a critical factor in high-demand scenarios like the COVID-19 pandemic. This integrated approach delivers safe, rapid, and scalable analysis, effectively meeting the demands of processing large sample volumes efficiently and cost-effectively. Backed by robust scientific evidence, this strategy is indispensable for ensuring timely and reliable diagnoses, significantly contributing to the effective management of public health emergencies. Incorporating agitators into RNA extraction protocols ensures uniform and effective extraction while enhancing the sensitivity and precision of molecular diagnostics, establishing itself as an essential practice in laboratories.

Similarly, the use of elevated temperatures, either alone or combined with lytic agents, is widely recommended to optimize viral RNA extraction, ensuring the accuracy and reliability of molecular diagnostic results. Thus, this combination of methods represents a practical, efficient, and validated solution for large-scale molecular diagnostic demands, reaffirming its crucial role in high-pressure laboratory contexts during public health emergencies.

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Ethical Approvals

The Research Ethics Committee of the Health Sciences Center at UFES approved this study (Study Protocol: 39187320.4.0000.5060). No human subjects were included in this study. The SARS-CoV-2 genetic material was isolated from nasopharyngeal samples of patients with suspected COVID-19 and was not linked to the donors.

Author Contributions

M. Palaci formulated and planned the study, facilitated the provision of facilities and necessary supplies, and provided guidance on modifications to the experiments. F.A.F. Martins and J.M. da Silva assisted with the experimental procedures, helping with the practical aspects and bench development. A. Rodrigues handled all the statistical aspects of the project, facilitating and enabling the development of the conclusion and discussion of the results. The final manuscript was reviewed and approved by all authors.

Conflicts of Interests

The authors declare that they have no conflicts of interest.

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