A simple RNA preparation method for SARS-CoV-2 detection by RT-qPCR

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Abstract

The technique RT-qPCR for viral RNA detection is the current worldwide strategy used for early detection of the novel coronavirus SARS-CoV-2. RNA extraction is a key pre-analytical step in RT-qPCR, often achieved using commercial kits. However, the magnitude of the COVID-19 pandemic is causing disruptions to the global supply chains used by many diagnostic laboratories to procure the commercial kits required for RNA extraction. Shortage in these essential reagents is even more acute in developing countries with no means to produce kits locally. We sought to find an alternative procedure to replace commercial kits using common reagents found in molecular biology laboratories. Here we report a method for RNA extraction that takes about 40 min to complete ten samples and is not more laborious than current commercial RNA extraction kits. We demonstrate that this method can be used to process nasopharyngeal swab samples and yields RT-qPCR results comparable to those obtained with commercial kits. Most importantly, this procedure can be easily implemented in any molecular diagnostic laboratory. Frequent testing is crucial for individual patient management as well as for public health decision making in this pandemic. Implementation of this method could maintain crucial testing going despite commercial kit shortages.

Keywords: Coronavirus; SARS-CoV-2; RNA extraction

Introduction

SARS-CoV2, a member of the *Coronaviridae* family, is the etiological agent of the current COVID-19 pandemic that has generated an international public health emergency. As of May 3rd, 2020, the virus has infected more than 3.3 million individuals and killed over 238,000 people worldwide (Situation Report 104 of the World Health Organization). Testing for the presence of the virus is of utmost importance for containment strategies aiming to reduce the dissemination of the virus and prescription of appropriate clinical practices for affected patients. However, understanding and managing the full extent of the outbreak has remained a challenge for most countries due to significant bottlenecks imposed by diagnosis[1].

Early detection of infection by SARS-CoV2 relies on the efficient detection of the viral genome using RT-qPCR. Several RT-qPCR-based tests are being used in clinical settings[2,] and novel approaches are continually being reported[3-10]. All methods require an RNA extraction step to isolate the viral genetic material before its detection. Unfortunately, RNA extraction has become a serious bottleneck for COVID-19 diagnosis around the world due to shortages in RNA-extraction kits customarily used to process patients samples. This is particularly troublesome in developing countries lacking the infrastructure and capacities to produce these kits locally. Before the kit-era, which contributed to standardize and simplify molecular biology work, several RNA extraction

methods were routinely used in research laboratories around the world. RNA isolation procedures typically involve three general steps: cell lysis, separation of RNA from other macromolecules such as DNA, proteins, and lipids, followed by RNA concentration. To prevent RNA degradation, cell lysis must be conducted under conditions that inhibit RNase activity, which is abundant in many cellular compartments[11,12]. RNA separation from other macromolecules is often achieved by a combination of pH and organic solvents, such as phenol/chloroform[13-16]. RNA concentration is most commonly achieved by high salt and isopropanol or ethanol precipitation[11, 12, 17-20].

We reviewed the published literature to search for procedures of RNA extraction that could potentially be used to replace commercial kits. Many different protocols and variations have been published over the years that optimize or simplify the RNA extraction process from various types of samples. We tested five types of procedures to identify an efficient procedure for extracting RNA from clinical samples that is compatible with downstream RT-qPCR analysis. Of the procedures evaluated, a simple method based on acid pH separation of RNA was found the most suitable one. It can be carried out in approximately 40 min for ten samples and is not more laborious than current methods using commercial kits. This procedure requires reagents and equipment that can be found in any standard molecular biology laboratory, thus avoiding supply chain issues. The resulting RNA can be used to detect SARS-CoV2 by standard RT-qPCR testing protocols with robust results comparable to those obtained using commercial RNA-extraction kits.

Results

Screening of alternative procedures for RNA extraction

For validation of the RNA extraction procedures, the RNase P target was amplified in a one-step RT-qPCR reaction, as quality control for the extraction method. As shown in **Figure 1**, three of the five procedures evaluated yielded enough RNA to amplify the RNase P target gene, whereas two of them did not. The Trizol approach was most effective, exhibiting the highest yield **(Figure 1)**. The BSA-based protocol also allowed for amplification of the RNase P target, albeit with lower yield and significant variability among replicates **(Figure 1)**. Acid pH-based method also allowed amplification of the target, though with lower yields when compared to the TRIzol method **(Figure 1)**. The direct method and high-temperature method did not yield enough RNA to amplify the RNase P gene under our experimental conditions. While TRIzol appears to be the best experimental procedure in terms of yield, it is not easy to use for a diagnostics laboratory setting as it requires a chemical hood for the organic extraction step. Biosafety cabinets class II (BSL-2) necessary for operator protection are not appropriate for working with organic solvents. BSA, Trizol, and acid-pH procedures provided comparable yields, but

the acid-pH method was more consistent among replicates. Based on these considerations, we decided to validate the acid-pH method to extract RNA from clinical samples, using the High Pure viral RNA extraction kit (Roche) as the gold standard.

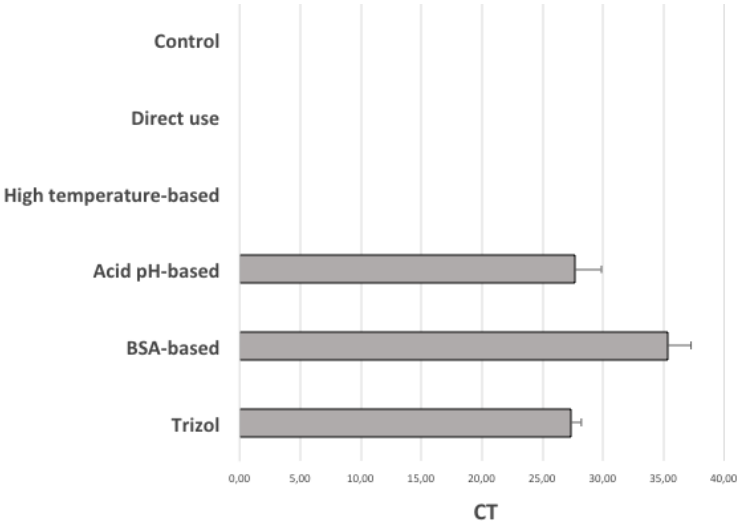

Figure 1. Quantitative assessment of performance for selected RNA extraction methods. CT values obtained by RT-qPCR with 45 cycles using TaqMan probe and primers against RNase P gene in saliva samples for Trizol (27.39 +/- 0.34), BSA-based (35.3 +/- 0.79), acid pH-based (27.68 +/- 0.90), high temperature-based (n.d.) and direct (n.d.) methods. n.d.; not determined (no CT reported). Control corresponds to a negative control with water instead of template. Bars show the mean plus standard deviation of the mean for two biological and three technical replicates each (6 measurements).

Validation of the acid pH RNA extraction method in clinical samples

In order to validate the acid pH method of RNA extraction, RT-qPCR using TaqMan probes and primers recommended by the CDC were used[33]. The nucleocapsid viral proteins N1 and N2 were amplified as viral targets, and RNase P was also amplified as a control. We analyzed 50 clinical samples: 22 were positive, 11 were undetermined, and 17 were negative according to RT-qPCR recommended by CDC using RNA extracted with HighPure viral RNA columns (Roche). Undetermined samples are described as having a viral load around the detection limit of the RT-qPCR method. The results for the 50 samples are shown in **Table 1**. The 17 negative samples were also negative using RNA extracted with the acid pH method. Out of 22 positive samples, 21 were also positive using RNA extracted with the acid pH method, whereas one sample was undetermined. Out of 11 undetermined samples analyzed, 4 were still undetermined using RNA extracted with the acid pH method. However, 3 of them were negative, and 4 of them were positive. The CT values for N1 and N2 obtained using Roche kit were

slightly higher on average than the values obtained using the acid pH method **(Figure 2A)**, but this difference was not statistically significant (p-value = 0.40 and 0.82 respectively). This analysis was done for positive and undetermined samples. The arithmetic difference between CT values obtained using Roche kit and acid pH method was on average positive for N1 and N2 targets (1.57 and 0.47 respectively) **(Figure 2B),** meaning that CTs were lower for the acid pH method. As shown in **Figure 2A**, CT values for the RNase P gene obtained using Roche kit were slightly lower than the average obtained by the acid pH method, but this difference was not statistically significant (p-value = 0.07). As expected, the difference between CT values was negative for RNase P (-0.95) **(Figure 2B),** meaning that CTs were slightly higher for the acid pH method. The % of agreement between acid pH method and Roche kit is 92%, considering as disagreement those samples whose report changed from positive to undetermined (sample 2882), and from undetermined to negative (samples 2946, 2943, and 3197). Importantly, the processing time and laboriousness of the acid-pH method is similar or less than that of Roche's HighPure columns method. A detailed scheme of the method is shown in **Figure 3**.

Table 1. Comparative data for the two RNA extraction methods tested.

	Commercial kit				Acid pH extraction method			
	N1	N2	RNase P	Report	N1	N2	RNase P	Report
2776*	37,57	38,19	27,91	positive	30,18	36,18	27,92	positive
2859*	18,93	16,72	31,64	positive	14,35	12,82	27,12	positive
2867*	38,51	39,18	26,1	positive	33,91	37,86	27,41	positive
2882*	**35,92**	**39,8**	**28,82**	**positive**	**36,73**	**41,97**	**32,17**	**undetermined**
3965	36,47	39,95	25,96	positive	34,43	38,95	26,68	positive
3211*	24,72	23,76	24,62	positive	23,79	26,99	27,49	positive
3413	35,45	38,36	26,13	positive	26,93	30,07	26,32	positive
3410	16,37	16,34	26,06	positive	15,62	16,31	27,96	positive
3426	24,12	26,16	24,74	positive	24,4	26,4	24,26	positive
3409	38,68	39,06	25,2	positive	31,4	34,96	25,44	positive
3865	19,93	20,82	26,18	positive	18,78	20,85	27,37	positive
3876	31,26	33,15	26,93	positive	30,49	35,51	27,82	positive
3879	35,14	36,84	25,89	positive	33,49	37,92	26,24	positive
3880	36,09	39,02	27,42	positive	34,91	41,8	27,92	positive
3911	16,92	17,76	24,34	positive	16,38	17,26	25,42	positive
3945	32,89	33,5	27,28	positive	32,45	37,75	27,68	positive
3976	17,74	19,97	28,42	positive	16,72	17,66	28,43	positive
3958	33,58	33,05	25,42	positive	35,28	37,97	28,37	positive
3959	21,17	18,65	27,53	positive	20,43	22,79	30,47	positive
4254	29,46	31,21	24,11	positive	30,74	31,29	24,63	positive
4210	31,85	38,51	25,31	positive	34,49	39,01	28,84	positive
4146	32,76	34,12	28,1	positive	29,94	31,65	27,51	positive
2946*	**38,95**	**42,31**	**28,11**	**undetermined**	**42**	**42**	**30,8**	**negative**
2943*	**39,96**	**42**	**26,52**	**undetermined**	**42**	**42**	**24,91**	**negative**
3197*	**37,93**	**41,99**	**25,98**	**undetermined**	**42**	**42**	**25,48**	**negative**
2815*	**39,25**	**41,2**	**31,14**	**undetermined**	**32,52**	**38,67**	**28,32**	**positive**
3231	**33,93**	**40,97**	**27,3**	**undetermined**	**32,24**	**36,81**	**29,4**	**positive**

3285	**36,61**	**40,03**	**26**	**undetermined**	**32,06**	**37,47**	**27,89**	**positive**
3298	**37,64**	**42**	**26,22**	**undetermined**	**32,07**	**36,86**	**35,89**	**positive**
3831	34,93	40,21	27,75	undetermined	37,09	**42**	29,76	undetermined
3471	38	41,94	30,3	undetermined	35,72	40,73	29,21	undetermined
3474	36,92	**42**	28,33	undetermined	35,93	41,71	28,14	undetermined
3479	38,91	**42**	30,28	undetermined	37,22	40,97	30,24	undetermined
2517	**42**	**42**	22,61	Negative	**42**	**42**	26,55	negative
2518	**42**	**42**	25,98	Negative	**42**	**42**	33,63	negative
2927*	**42**	**42**	29,55	negative	**42**	**42**	27,76	negative
3877	**42**	**42**	29,21	negative	**42**	**42**	28,97	negative
3878	**42**	**42**	27,11	negative	**42**	**42**	26,31	negative
3881	**42**	**42**	26,07	negative	**42**	**42**	25,95	negative
3882	**42**	**42**	25,13	negative	**42**	**42**	24,19	negative
3973	**42**	**42**	29,03	negative	**42**	**42**	30,24	negative
3960	**42**	**42**	25,8	negative	**42**	**42**	24,94	negative
3961	**42**	**42**	29,96	negative	**42**	**42**	31,62	negative
3962	**42**	**42**	29,15	negative	**42**	**42**	33,11	negative
3963	**42**	**42**	26,82	negative	**42**	**42**	28,16	negative
3964	**42**	**42**	29,18	negative	**42**	**42**	32,58	negative
4170	**42**	**42**	29,85	negative	**42**	**42**	34,6	negative
4173	**42**	**42**	27,25	negative	**42**	**42**	32,82	negative
4174	**42**	**42**	28,4	negative	**42**	**42**	32,92	negative
4175	**42**	**42**	29,63	negative	**42**	**42**	33,72	negative

The * denotes extraction was done with 600 µL of Lysis Buffer. All other samples were extracted using 300 µL as described in Materials and Methods. Bold letters show samples that changed their report's results. A CT value of 42 was considered for those negative q-PCR results where no CT value is provided in order to calculate the difference between CT values.

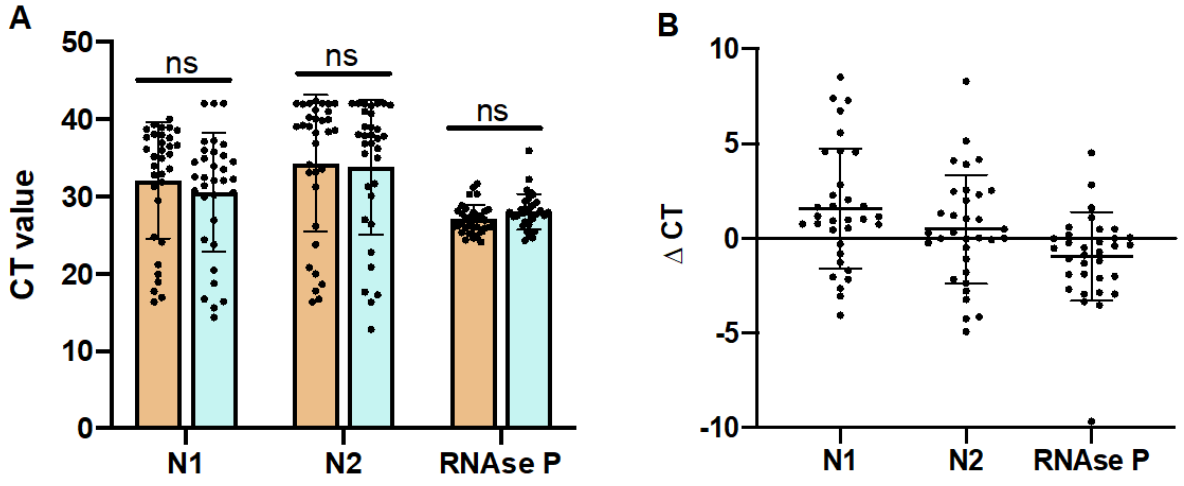

Figure 2. **The acid-pH method provides comparable results to commercial kits in clinical samples**. (A) Each bar represents the mean +/- standard deviation CT values for each RT-qPCR target fragment N1, N2, and Rnase P. Orange bars show results obtained with HighPure kit (Roche). Blue bars show results obtained with the acid-pH method. (B) Graph representing the difference between CTs obtained through both methods. ΔCT was calculated for each sample as CT obtained with High Pure column minus the CT obtained with acid pH RNA extraction method.

The graph shows data for positive and undetermined samples (n=33). The data were analyzed by a Student's t-test. 'ns' means no statistically significant differences.

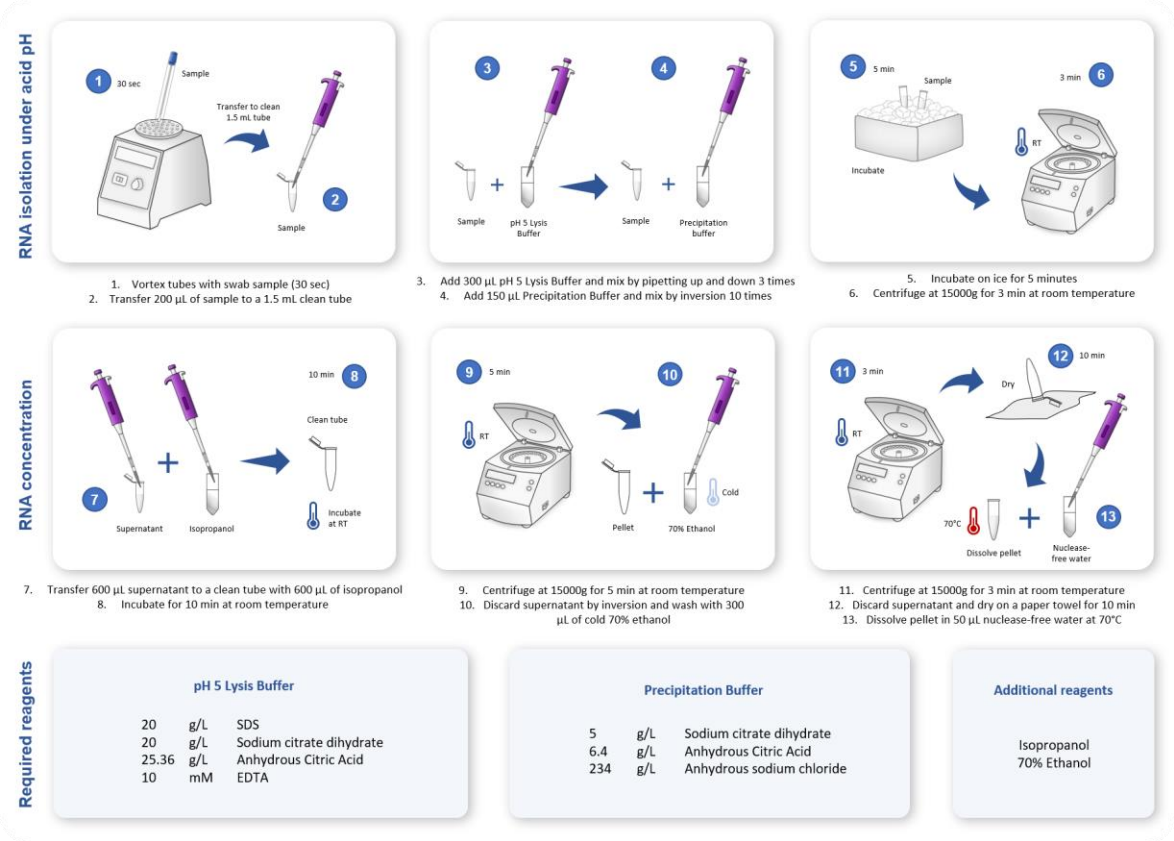

Figure 3. Schematic diagram of the validated acid-pH method for RNA extraction compatible with SARS-CoV2 RT-PCR testing. Steps carried out in the acid pH RNA extraction protocol.

Discussion

Here we tested several kit-free RNA extraction methods compatible with RT-qPCR analysis and selected one simple procedure based on RNA extraction using acid pH. We validated this method using 50 clinical samples with results comparable to those obtained with commercial kits. There are three key aspects of this method that must be pointed out. First, the acid pH-based methods that we reviewed[11,15,21] are intended for RNA extraction from tissue, cultured cells, and cell-associated virus. Therefore, the first step of these protocols is centrifugation with subsequent lysis of the cell pellet. However, we need to recover free viral particles in solution, which do not sediment after routine centrifugation at 15,000 g. For this reason, we used the uncentrifuged sample directly mixed with lysis buffer, with subsequent precipitation of viral RNA in the whole mix volume. Using uncentrifuged sample is the critical step for efficient RNA recovery

because when a centrifuged sample was used in preliminary tests, CT values were much higher than those obtained with Roche kit. Second, the acid pH method uses the anionic detergent Sodium dodecyl sulfate (SDS) that can lyse cells and viral coats through disruption of noncovalent bonds in proteins, causing them to lose their native conformation[11]. Third, low pH and high concentration of salt make possible the selective recovery of RNA. Within the pH range of 5.5 to 6.0, RNA degradation is minimized[21]. RNA phosphodiester bond is more stable at acidic than alkaline pH, where it is susceptible to alkaline hydrolysis at pH greater than 6[22]. Acid hydrolysis can only occur at pH lower than 2[23,24]. Moreover, DNA and RNA have different solubility at different pH, mainly due to the 2' hydroxyl group of RNA, which increases the polarity of this nucleic acid[25,26].

It is worth mentioning that all of the samples that changed their report had CT values that were around the cutoff value of 40. These changes occurred in both directions, meaning that some CTs increased and some CTs decreased. It would have been very clarifying to perform triplicated RNA extractions, in particular for undetermined samples, whose viral load is around the detection limit. Because of the above-exposed information, we consider the acid pH method robust and reliable. In fact, it is currently being used in our diagnostic laboratory since the 3rd week of April 2020 for routine detection of SARS-CoV2 in clinical samples.

The RNA extraction procedure with acid pH described here has many advantages over commercial kits to test for SARS-CoV-2 in the context of the current pandemic. This experimental procedure utilizes low-cost reagents and equipment that can be found in standard molecular biology laboratories. The cost of extraction is a critical issue in most clinical laboratories, and the cost of our in-house method is around ten times lower than extraction kits. Moreover, DNase treatment is not necessary because SARS-CoV-2 detection is not altered in the presence of DNA. Residual DNA may serve as the template for RNase P gene amplification. Because of current environmental concerns, we would also like to highlight the lower plastic contamination generated by this in-house method. Column-based extraction kits use several disposable tubes per sample, columns, bottles of buffer solutions, and plastic bags. Our in-house extraction method is, by far, much more environmental friendly; it requires only two eppendorf tubes per sample. Finally, our in-house method is comparable in hands-on time to commercial kits: it can be carried out in approximately 40 minutes for a set of 10 samples.

In conclusion, the RNA extraction procedure with acid pH described here is an excellent alternative to commercial systems to test for SARS-CoV2. Our results support a new method for RNA extraction from swab samples that can be used to detect SARS-CoV2 by standard RT-qPCR testing protocols. This procedure can be a helpful alternative for laboratories facing supply-chain disruption and commercial kit shortages.

Materials and Methods

Biological samples

Two types of biological samples were used. For preliminary evaluation of the RNA extraction methods, we used saliva samples obtained from two asymptomatic volunteers. Saliva is routinely collected for the initial assessment of viral infection. Two saliva samples were obtained from each volunteer, and at least three independent RNA extractions were performed from each sample, obtaining a minimum of six RNA preparations to test each experimental procedure. For validation of the RNA extraction method selected, we used nasopharyngeal swabs in Universal Transport Medium (UTM). Swabs were obtained from 50 patients that attended the outpatient service of Red Salud UC-CHRISTUS (Santiago, Chile) because of suspected coronavirus infection. Samples were processed in the Laboratory of Diagnostic Microbiology of the same institution using standard procedures that were approved by the Ethics Committee of the Pontificia Universidad Católica de Chile.

RNA extraction methods evaluated

The following experimental procedures were tested in this study. Saliva samples were centrifuged before taking an aliquot of supernatant for processing, as described below.

(1) TRIzol. The standard TRIzol-based method was evaluated[8,12,19]. First, 800 µL of TRIzol were added to 200 µL of sample and vortexed briefly. Then, 200 µL of chloroform were added, vortexed, and centrifuged at 12,000 g for 10 min at room temperature. The aqueous phase (600 µL) was recovered in a clean tube containing 600 µL of isopropanol. The tube was mixed by inversion and incubated at room temperature for 10 min. The tube was then centrifuged at 12,000 g for 10 min at 4°C, and the supernatant was discarded. The pellet was washed with 500 µL of 70% ethanol, centrifuged at 7,500 g for 5 min at 4°C and the supernatant was discarded. The pellet was dried at room temperature for 10 min and resuspended in 25 µL of RNase-free water by incubating at 37°C for 10 min.

(2) BSA-based method. Previous reports show that BSA has positive effects on RT-qPCR results when added to samples in the presence of inhibitors[27,28]. Based on the procedure described by Plante *et al.* (2010)[27] and Svec *et al.* (2013)[28], a 200 µL aliquot sample was centrifuged at 12,000 g for 30 s at room temperature. Then, 2.5 µL of supernatant were added to 47.5 µL of a 1 mg/mL BSA solution (1:20 ratio), vortexed for 30 s and kept on ice or at −80°C until further use.

(3) Acid pH-based method. Under acidic pH, RNA can be separated from DNA and other molecules due to the differential polarity given by its hydroxyl groups, which maintains it in solution[11,29,21,25,26]. Based on the methods described by Heath (1999) [21],

Sambrook and Russell (2001) [11], and Chomczynski and Sacchi (2006) [15], 300 µL of pH 5 Lysis Buffer (20 g/L sodium dodecyl sulfate (SDS), 20 g/L sodium citrate dihydrate, 25.36 g/L anhydrous citric acid and 10 mM EDTA) were added to 200 µL of uncentrifuged sample and mixed by pipetting three times. Then, 150 µL of Precipitation Buffer (5 g/L sodium citrate dihydrate, 6.4 g/L anhydrous citric acid, and 234 g/L anhydrous NaCl) were added and mixed by inversion 10 times. Samples were incubated on ice for 5 min and centrifuged at 15,000 g for 3 min at room temperature. Six hundred µL of the supernatant were transferred to a clean tube containing 600 µL of isopropanol and incubated for 10 min at room temperature. A new centrifugation step was made at 15,000 g for 5 min at room temperature. The supernatant was removed, and the pellet washed with 300 µL of cold 70% ethanol and centrifuged at 15,000 g for 3 min at room temperature. The supernatant was discarded, and tubes were inverted in a paper towel. The pellet was dried, leaving the tubes open for 10 min. Finally, the pellet was resuspended in 50 µL of nuclease-free water pre-warmed at 70°C.

(4) High temperature-based method. Based on the method described by Fomsgaard and Rosenstierne (2020)[30], 50 µL of the sample were directly heated at 98°C for 5 min and cooled at 4°C. Then 19 µL of the sample were mixed with 1 µL of BSA (20 mg/mL) and kept on ice for immediate use or at -80 °C for later use.

(5) Direct use of the samples. An aliquot taken from the original sample was directly used to perform RT-qPCR analysis[31].

The 50 nasopharyngeal swabs used for the validation of the RNA extraction method selected were extracted using the High Pure viral RNA extraction kit (Roche) according to instructions provided by the manufacturer. This RNA extraction method was considered as the gold standard for comparison purposes, and It is based on the capture of RNA using columns with silica filters.

RT-qPCR analysis

For preliminary evaluation of RNA extraction procedures, we used RT-qPCR against the human RNase P gene with primers and a TaqMan probe previously described[32]. Five µl of RNA from saliva samples, 2 µl of RNase-Free water, 1 µl of each RNase P primer, 1 µl of TaqMan RNase P probe and 10 µl of 2X TaqMan Fast Universal PCR Master Mix (Applied Biosystems), were used for RT-qPCR in a final reaction volume of 20 µL performed in a StepOnePlus Real-Time PCR System (Applied Biosystems).

For validation of the selected RNA extraction procedure, TaqPath COVID-19 RT-qPCR (Applied Biosystems) using TaqMan probes and primers recommended by the CDC was used[33]. Two viral targets were amplified from RNA obtained from nasopharyngeal swabs

samples: the nucleocapsid viral proteins N1 and N2. The RNase P target is also amplified as quality control for the extraction method and to corroborate the absence of PCR-inhibitors in the sample. A one-step RT-qPCR reaction was performed in a StepOnePlus Real-Time PCR System (Applied Biosystems). Cutoff points for CT values (Cycle Threshold) required to decide whether a result is COVID-19 positive or negative were those specified by the CDC as follows. To report a positive result, both viral targets N1 and N2 must be CT<40. To report a negative result, both viral targets must be CT≥40. If one of the viral targets is CT<40, and the other is CT≥40, the result must be reported as undetermined. The RNase P target must be CT≤35.

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Author Contributions
AW performed experimental work, data analysis, figure composition, and manuscript writing. AC, CIH, and VS did experimental work, data analysis, and figure composition. GA, LL, performed a literature search and manuscript writing. CM, ML, SS, AMG, and TQ contributed to experimental design and critical data analysis. SH, ER, MF, performed a literature search and defined protocols for testing. RAG and PG performed the literature search, experimental design, data analysis, and manuscript writing. They co-supervised this project.

Additional Information
The authors declare no competing interests.

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