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Forensic evaluation of two nucleic acid extraction systems and validation of a RT-qPCR protocol for identification of SARS-CoV-2 in post-mortem nasopharyngeal swabs

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\section*{Abstract}

The COVID-19 outbreak has represented a challenge for the international scientific community and particularly for forensic sciences. The lack of Coronavirus post-mortem testing led the National Institute of Toxicology and Forensic Sciences (INTCF) from Spain to verify the performance and utility of a quantitative reverse transcription PCR (RT-qPCR) clinical diagnosis protocol for SARS-CoV-2 detection (TaqPath\textsuperscript{TM} COVID-19 CE-IVD RT-PCR Kit), to shed light on the cause of death (COD) in potentially COVID-19 cases in judicial autopsies. Two different RNA extraction methods were also tested (EZ1\textsuperscript{®} Advanced XL robot versus MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit) regarding extraction efficiency, precision and contamination. RT-qPCR was evaluated for precision, specificity, limit of detection and concordance. Both the automated and the manual RNA extraction procedures showed good efficiency, but the automated virus extraction by bio-robot produced more reproducible results than the manual extraction. The SARS-CoV-2 RT-qPCR assay showed high sensitivity with a detection limit up to 10 copies/reaction and high specificity, as no cross-reactivity was detected between any of the 12 different RNA viruses tested, including three types of coronaviruses (SARS-CoV, NL63 and 229E). Reproducibility and repeatability of the studied method as well as concordance with other SARS-CoV-2 molecular detection protocols were also demonstrated.

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1. Introduction

The disease caused by the novel Coronavirus SARS-CoV-2 (Severe Acute Respiratory Syndrome Coronavirus 2), also called COVID-19 (Coronavirus Infectious Disease-19), represents a cluster of pneumonia that emerged at the end of 2019 in the Chinese city of Wuhan, Hubei province, from where it spread to China and other countries in the world [1–5]. The COVID-19 was declared by the World Health Organization (WHO) as a Public Health Emergency of International Concern (PHEIC) on January 30, 2020 [6], and was characterized as a pandemic on March 11, 2020 [7].

The availability of the complete genome of SARS-CoV-2 early in the epidemic [8], allowed the development of specific primers, targeting the viral genes (ORF1ab, E, S and N), to amplify the genetic material of SARS-CoV-2 by using the Reverse Transcription Quantitative Real Time Polymerase chain reaction (RT-qPCR).

Initially, several laboratories developed many in house molecular tests but shortly after different commercial assays started to appear. Currently there are more than 240 molecular assays to diagnose COVID-19, with CE-IVD certification [9], among them the TaqPath\textsuperscript{TM} COVID-19 CE-IVD RT-PCR Kit (Thermo Fisher Inc., CA, USA) [10]. Other nucleic amplification/detection methods such as CRISPR or LAMP (loop-mediated isothermal amplification) are in the process of being commercialized [11–14]. The WHO recommends molecular testing (RT-qPCR) as a confirmatory diagnostic test [15,16], although laboratories undertaking COVID-19 virus testing should perform a previous validation and both external and internal controls PCR should be included to provide a reliable test result.

Since the beginning of the coronavirus pandemic, clinical laboratories have been validating and implementing molecular tests...
with the aim of establishing reliable COVID-19 diagnosis in patients and contacts. However, confirmation of COVID-19 as cause of death (COD) in previously undiagnosed patients, either at home or in hospitals, has not always been possible: the lack of adequate safety requirements has led to autopsy restrictions and therefore to underdiagnosis.

In the light of the importance of autopsy findings for understanding COVID-19 features and the establishment of the COD, the Instituto Nacional de Toxicología y Ciencias Forenses (INTCF, National Institute of Toxicology and Forensic Sciences), a technical body attached to the Ministry of Justice of Spain [17], decided to validate and implement a protocol for extracting and detecting the genetic material of the SARS-CoV-2 in post-mortem specimens from judicial autopsies. In this work, we present the results obtained from the performance characteristics studied.

2. Materials and methods

2.1. Samples/controls, RNA controls and experimental designs

Extraction efficiency, repeatability and reproducibility were conducted using the AMPLIFIRE® TOTAL SARS-CoV-2 CONTROL (SWAB) (Vircell Microbiologists, Granada, Spain) [18], Integral Control of the Process of Extraction and detection (hereinafter CPE), which simulates swabs. From this control, sub-samples were prepared to be extracted with 2 different known (and theoretical) viral copies (Genome Copy Equivalent, GCE) inputs at the end point (in the RT-qPCR reaction) of high and medium GCE (500 and 250 total copies). The GCE value is “theoretical” based on the value provided by the manufacturer. The CPE control was extracted/detected in triplicate within each extraction batch, for the total GCE inputs per reaction, for each extraction system, and detected on two plates (i.e., 2 replicates × 2 inputs × 2nd extraction batch = 8 samples/controls for each extraction system × 2 extraction systems = 24 samples/controls × 2 detection plates = 48 samples/controls in total).

Repeatability test was also performed in triplicate with a quantified control of SARS-CoV-2 RNA (10,000 copies/µL provided by the Respiratory Viruses and Flu Unit of the National Center for Microbiology of the Carlos III Health Institute (CNM-ISCIII, Majadahonda, Madrid, Spain). This control was introduced with a known final input of 5000 copies/reaction, and whose replicates were detected on two plates (i.e., 6 samples total).

Concordance was assessed using five eluates of nasopharyngeal swabs from SARS-CoV-2 positive patients. These samples were previously analyzed at the Clinical Microbiology Service of the San Carlos Clinical Hospital (HCSC, Madrid, Spain). These eluates were divided into 2 aliquots (1 for each extraction system). One aliquot of each of these 5 samples was extracted by both extraction systems and detected on 2 plates (i.e., 5 × 2 samples × 2 detection plates = 20 samples). Once analyzed by RT-qPCR, the Cts values obtained were compared between the extraction system and with the results obtained in the HCSC.

Specificity studies were drawn from 12 different RNA viruses extracts to assess possible cross-reactivity. The AMPLIFIRE® CORONAVIRUS SARS (2003) RNA CONTROL (Vircell Microbiologists, Granada, Spain) (hereinafter COV1 control) [19] was used, RNA purified from the Coronavirus SARS-CoV-2 (2003), of which two known inputs were analyzed: 5000 and 50 copies/reaction. Additionally, other respiratory viruses from the Proficiency Testing Nucleic acid Amplification, Respiratory (ID2) del College of American Pathologists (CAP) [20] were analyzed, including: two of the most frequent Coronaviruses, two extracts of Coronavirus NL63 (2017-ID2-06 and 2019-ID2-06) and one extract of Coronavirus 229E (2018-ID2-06); eight extracts of Influenza A virus H1N1/2009 (2018-ID2-01), Influenza A virus H3N2 (2019-ID2-01), Human metapneumovirus B (2019-ID2-05), Influenza B virus (2019-ID2-07), Parainfluenza virus 2 (2019-ID2-08), Respiratory syncytial virus (2019-ID2-09), and Rhinovirus (2019-ID2-12). All extracts were analyzed in duplicate on each of the two detection plates (i.e., 12 virus RNA × 2 replicates × 2 detection plates = 48 viruses RNA in total).

Limit of detection (sensitivity) testing was conducted by using 5 serial dilutions (10,000; 5000; 500; 50 and 10 copies/reaction) of two SARS-CoV-2 RNA controls: the AMPLIFIRE® CORONAVIRUS SARS-CoV-2 RNA CONTROL (21) (hereinafter C19 control), with an initial concentration of 14,500 copies/µL, and the TqPath™ COVID-19 Control (10) (hereinafter CTP control), with an initial concentration of 10,000 copies/µL. The last dilution, 10 copies/reaction, is the limit established by the manufacturer [10]. Each dilution was analyzed in duplicate on two plates (i.e., 5 serial dilutions × 2 replicates × 2 controls × 2 detection plates = 40 controls in total).

To evaluate potential external or cross-contamination, eight negative extraction controls were analyzed. In each extraction batch 2 negative controls were incorporated (2 CN_EXT × 4 extraction batch = 8 CN_EXT), which were positioned so that they were close to those samples that contained the greatest number of copies.

The MS2 Phage Control, included in the TqPath™ COVID-19 CE-IVD RT-PCR Kit (Thermo Fisher Inc., CA, USA) (hereinafter TPkit) [10], was added to the samples before extraction of the RNA, according to the manufacturer’s recommendation [10]. The MS2 Phage Control allowed to verify the efficacy of the sample preparation and the absence of inhibitors in the PCR reaction [10]. By this way, the generic evaluation of this control was performed considering the Cts values obtained for the MS2 target of the RT-qPCR in each of the samples analyzed in this verification: different inputs of the CPE control [18]; clinical samples provided by the HCSC; negative extraction controls for each batch for each of the extraction systems evaluated.

2.2. Viral nucleic acids extraction

Viral nucleic acids from samples and controls were extracted with two different extraction systems according to the manufacturer’s recommendation: (1) one automated, the EZ1® DSP Virus Kit [22] on the EZ1® Advanced XL robot [23] (Qiagen, Hilden, Germany) (hereinafter QEZ1); (2) another manual, the MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit (Thermo Fisher Inc., CA, USA) [24,25] (hereinafter TFS-MM). For both extraction systems, the following parameters were verified extraction efficiency, repeatability, reproducibility, contamination and concordance.

The extraction efficiency was evaluated through a “relative recovery index”, which provides a relative percentage value of the extraction efficiency between both systems. Based on the GCE value obtained for each theoretical input and the expected GCE value, this recovery index was calculated as follows: RI% = (GCE obtained) / (GCE expected).

2.3. SARS-CoV-2 nucleic acids detection

The TqPath™ COVID-19 CE-IVD RT-PCR Kit (Thermo Fisher Inc., CA, USA) [10] was verified for the qualitative detection of nucleic acid from SARS-CoV-2. This kit with CE-marked In Vitro Diagnostics (CE-IVD) allows the virus detection in upper respiratory specimens (such as nasopharyngeal, oropharyngeal, nasal, and mid-turbinate swabs, as well as nasopharyngeal aspirate) and bronchoalveolar lavage (BAL) specimens from individuals suspected of COVID-19. In this kit, probes anneal to three target sequences that are specific to SARS-CoV-2, reducing the risk of detecting other coronaviruses. Each target is located between unique forward and reverse primers for the following genes: ORF1a/b (open reading frame), N Protein (nucleocapsid protein) and S Protein (spike protein). All analyses were carried out on the QuantStudio 5 Real-Time PCR Systems
3. Results and discussion

3.1. Viral nucleic acids extraction

In determining the most appropriate extraction method for obtaining the SARS-CoV-2 genetic material, the following results were obtained for the parameters evaluated:

3.1.1. Extraction efficiency

The evaluation of this parameter was performed with the CPE [18], at two known (and theoretical) inputs of copies per reaction (GCE). Based on these theoretical values, the relationship between the two extraction systems was assessed (Table 1). Overall, the automatic method QEZ1 [22] was found to be more than 25% efficient for each probe compared to the manual method TFS-MM [24,25]. These differences were greater the lower the GCE of the sample (for theoretical 250 GCEs, the differences between both systems would slightly exceed 29%, except in the case of the S gene probe). Considering the Cycles threshold (Cts) values, instead of the relative copy number value, the intra- and inter-system variability would be lower, taking into account the variability coefficient (Supplementary Table S1).

3.1.2. Repeatability

Repeatability was assessed with the CPE [18], for each extraction system and two different operators. In Supplementary Table S2, the results for each extraction system detected in two RT-qPCR plates are shown. For the two extraction batches performed for each system and detected in duplicate by RT-qPCR, in the three viral regions analyzed, a repeatability of 100% was obtained at a qualitative level.

In addition, it can be indicated that the average values of the coefficient of variation (CV%) for the quantitative data of Cts values did not exceed 3.5% (Supplementary Table S2). This fact shows that both extraction systems, at a quantitative level, have good repeatability, slightly better in the case of the automatic system QEZ1 [22].

3.1.3. Reproducibility

Reproducibility was assessed with the CPE [18], between extraction batches carried out by different operators for each extraction system. In the Supplementary Table S3, the results obtained in the two RT-qPCR detection plates are shown, in which the results were analyzed in duplicate. For the two extraction batches performed in each system and detected on both RT-qPCR detection plates, for the three regions analyzed, a reproducibility of 100% was obtained at a qualitative level.

For the quantitative data of Cts values, the average values of the CV% did not exceed 5%. At the quantitative level (based on Cts values), statistically significant differences were observed between both extraction batches for the manual system TFS-MM [24,25] in both RT-qPCR detection plates for each viral region analyzed (Supplementary Table S3). This fact indicates that, although the reproducibility was 100% at the qualitative level (positive/negative results), the variability of this extraction system was statistically significant at the quantitative level. The clearest explanation is that it is a manual extraction system, with what this entails, and, although there may be quantitative differences between operators, these differences would not have any repercussions at a qualitative level. Consequently, the automatic extraction system QEZ1 [22] would be more robust, with no statistically significant differences at the quantitative level.

3.1.4. Contamination

In Supplemental Table S4, the contamination study data are collected for each extraction batch and each system on each of the RT-qPCR detection plates run. Generally, the analysis of negative controls for both extraction systems detected in duplicate showed undetermined Ct values. Only in two of the probes a Ct result was detected (Supplementary Table S4, probe values in red). Nevertheless, these values can be considered as artifactual as they are not reproduced in the rest of the probes of the same negative extraction control (see images included in Supplementary Table S4). And the linear representation of the amplification plots for these logarithmic artifactual curves shows that there was no amplification process. Therefore, they should be considered as negative results at a qualitative level, according to the manufacturer’s recommendations [10].

### Table 1

|  | 500 GCE | 250 GCE |
|---|---|---|
| ORF1ab | N gene | S gene | ORF1ab | N gene | S gene |
| QEZ1/TFS-MM Index | 0.997 | 1.199 | 1.873 | 2.052 | 1.299 | 0.872 |
| Average QEZ1/TFS-MM index | 1.525 | 1.249 | 1.372 |

Indices are collected between the automatic extraction system EZ1® DSP Virus Kit (QEZ1) and the manual system MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit (TFS-MM) for each of the probes analyzed with the TaqPath™ COVID-19 CE-IVD RT-PCR Kit. These indices have been calculated for two theoretical inputs of the AMPURiON® TOTAL SARS-COV-2 CONTROL. The averages of this index are collected for each input and for the set of both in each probe.
3.1.5. Concordance

In the Supplementary Table S5, the concordance results are collected for both extraction systems, for each of the RT-qPCR detection plates. As indicated (see Section 2.3.), the commercial TPkit [10] allows detecting three regions of the SARS-CoV-2. However, for the clinical samples previously analyzed in the HCSC, only the previous result of one of the genes (protein N gene) was available. For the clinical samples analyzed with each extraction system and detected in both RT-qPCR detection plates, the concordance for the protein N gene was 100% at a qualitative level, according to the HCSC’s results.

3.1.6. Internal extraction control (phage MS2)

The evaluation of the phage MS2 is essential due to its importance in the extraction process (as an efficiency control of this process). In Supplementary Table S6, the raw data of the Cts values obtained for each of the samples analyzed in this verification are collected: different inputs of the CPE control [18]; clinical samples provided by the HCSC; and negative extraction controls. In addition, at the Supplementary Table S6, the general statistical data (mean, standard deviation and coefficient of variation) of the Cts obtained for the probe MS2 are shown, for each extraction system in each detection plates of RT-qPCR, as well as for the joint data from both plates. Likewise, the expected Cts range for this MS2 probe is collected, calculated as the mean of the Cts values plus/minus 2 standard deviations (95% confidence interval) [35].

For the MS2 probe, the average variability between both extraction systems presented differences of almost double, this CV% being greater in the case of the manual system TFS-MM [24,25]. After removing outlier values, the average ranges of expected Cts for the MS2 probe obtained for each type of extraction was 25.92–29.77 Cts, in the case of the QE1 extraction, and 25.77–31.22 Cts, in the case of the TFS-MM extraction. These ranges allow the quality parameters of the RT-qPCR detection system to be established, under laboratory conditions for each extraction system.

3.2. SARS-CoV-2 nucleic acids detection

3.2.1. Selectivity/specificity

During the developmental validation of the TPkit [10], in silico analysis of 43 microorganisms was performed. Although blast analysis showed ≥80% homology for one assay component (forward primer, reverse primer, or probe) for select isolates, there would be no anticipated amplification because hybridization of all three assay components are necessary to generate a signal [10]. The in silico analysis indicated that significant amplification of non-target sequences that result in cross-reactivity or potentially interfere with detection of SARS-CoV-2 was not likely to occur [10].

In this study, 12 real extracts from different RNA viruses were analyzed, including three types of coronavirus (SARS, NL63 and 229E). All these extracts were amplified in duplicate on two different detection plates. In the Supplementary Table S7, the specificity results of the TPkit [10], for each RT-qPCR detection plate shows that there was no cross-reaction. Although, some samples presented Cts values, these values can be considered as artifactual as they were not reproduced in the rest of the probes of the same negative extraction control (see images included in Supplementary Table S7). And the linear representation of the amplification plots for these logarithmic artifactual curves showed that there was no amplification process. Therefore, they should be considered as negative results at a qualitative level, according to the manufacturer’s recommendations [10].

![Fig. 1. Serial dilutions detection of the AMPLIFUN® CORONAVIRUS SARS-CoV-2 RNA CONTROL using the TaqPath™ COVID-19 CE-IVD RT-PCR Kit. Images from the Design & Analysis Software v2.3.3.](https://example.com/fig1.png)

A: Standard curves for each of the probes analyzed (ORF1ab, protein N and protein S genes) and their parameters (slope, R2, Y-inter, Eff% and error).
B: Serial dilutions amplification curves of the N protein gene probe.
C: Serial dilutions amplification curves of the ORF1ab gene probe.
D: Serial dilutions amplification curves of the protein S gene probe.
3.2.3. Repetitability

Those samples used for extraction repeatability were assessed in the detection repeatability (Supplementary Table S2). Additionally, three replicates of a quantified control of SARS-CoV-2 RNA provided by the CNM-ISCIII were evaluated (Supplementary Table S9). Overall, a repeatability of 100% was obtained at a qualitative level.

For the quantified control of RNA of the SARS-CoV-2 provided by the CNM-ISCIII, the average values of CV% for the quantitative data of Cts values did not exceed 1.5% (Supplementary Table S9) in each probe tested, consistent with the values obtained for the CPE control (Supplementary Table S2).

3.2.4. Reproducibility

The relative differences between the two RT-qPCR detection plates, performed on the same Q5S kit at two different times, were assessed. Supplementary Table S10 shows the results obtained for both RT-qPCR detection plates. The intra-group variability of samples did not exceed 20% (CV%) for each probe testes. Within each RT-qPCR detection plate, for each probe analyzed, the CV% did not exceed 15%.

Assessing the t-Student contrast statistic for the set of both RT-qPCR detection plates, no statistically significant differences between plates were observed, for each probe analyzed (Supplementary Table S10). In any case, making this assessment by group of samples, only statistically significant differences were observed for the quantified control of RNA of the SARS-CoV-2 provided by the CNM-ISCIII, but only for the N gene probe. These differences can be due to the fact that in this case only 3 controls were available, despite the t-Student statistic being indicated for small sample sizes. In any case, as indicated, the TPkit [10] is a qualitative not quantitative detection system, therefore the inter-plate reproducibility would be 100% at a qualitative level.
manual extraction method TFS-MM [24,25] meets many of the requirements, the automatic extraction method QEZ1 [22] seems to be the most appropriate for the workflow of our laboratory.

Regarding the detection method, sensitivity and specificity, together with the low percentage or null of false positives and negatives, will be fundamental parameters to assess the utility of any diagnostic test and its predictive value [36,37]. In the case of post-mortem samples, this predictive value will be relative since, except for anatomopathological studies, it will be difficult to confirm whether the RT-qPCR result is correct or is a false negative or positive result. As Watson et al. point out [38], no test gives a 100% accurate result and there is no “gold standard” test to compare with, so evaluating the accuracy of any test, even if it is RT-qPCR, will be challenging. Higher sensitivities are reported depending on which gene targets are used, and whether multiple gene tests are used in combination [39,40].

Regarding the efficiency/specificity of the TPkit [10], it is important to highlight that its multi-target design (simultaneous analyses of three regions of the genome: ORF1ab, protein S and protein N genes), could allow to detect new variants of the SARS-CoV-2, such as the one discovered in the United Kingdom (B.1.1.7 lineage, S0Y1V1 variant) [41], already present in Spain. This variant has a specific mutation in the S-gene which results in a deletion of two amino acids at sites 69 (histidine) and 70 (valine), commonly referred to as 69–70del [41]. In this case, even if the mutation in the S-gene could produce a negative result in the protein S target (S-gene dropout), the other two probes results of the TPkit [10], reduce the number of false negatives (and/or positives), by requiring that at least two probes be positive to give a final positive result. Additionally, this kit includes an internal control of the extraction process, the phage MS2, which allows monitoring the entire process, from the efficiency of RNA extraction to the detection of possible amplification inhibitors.

Nevertheless, The European Centre for Disease Prevention and Control (ECDC) notes that, “Laboratories should review the PCR performance and drop-out of the S-gene. PCR could be used as an indicator for cases with the new variant for further sequencing and investigation” [42].

On the other hand, an important point in the accuracy of the detection method by RT-qPCR is that it probably also varies according to the stage of the disease [43] and the degree of viral multiplication or clearance of the virus [44]. In the case of post-mortem samples, this fact may have relevant consequences, since it is not possible to repeat the analyses at a later stage of the disease. Therefore, the sensitivity of the detection method will be essential. The TPkit [10] has very good sensitivity, up to 10 copies/reaction, which will mean up to 800 copies of the SARS-CoV-2 genome if it starts from a 400µL sample, which is the initial volume allowed by the automatic extraction method QEZ1 [22].

4. Conclusions

The current health emergency caused by the COVID-19 pandemic has made it necessary for forensic genetics laboratories to contribute their experience in the molecular biology field. With this perspective, the INTCF made the decision to develop an integral workflow for the extraction and detection of genetic material from the SARS-CoV-2 with very specific purposes, fundamentally, to confirm the presence of the virus in suspicious post-mortem samples and to help establish the COD in judicial autopsies.

The validation presented in this work shows that, the systems evaluated for the SARS-CoV-2 nucleic acids extraction from nasopharyngeal swabs are repetitive, reproducible and concordant with the automatic system showing slightly greater efficiency than the manual one. Regarding the virus detection system, the assessed kit is specific, sensitive, reproducible and concordant, presenting an acceptable predictive value for the required use.

The integral workflow for detecting the SARS-CoV-2 developed at INTCF is optimal for the required purpose. Furthermore, it allows a complete integration with the laboratory system, integrating the entire process with the internal LIMS system, from the generation of the samples to the generation of the final results report.

Conflict of interest statement

None declared.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.forsciint.2021.110775.

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