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SHORT COMMUNICATION

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Development and implementation of a RT-qPCR extraction-free protocol for the detection of SARS-CoV-2 and impact on the turn-around-time

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Abstract

The occurrence of the COVID-19 second-wave outbreak in Europe has pushed laboratories performing molecular SARS-CoV-2 tests to increase their throughput and decrease the result rendering time. In this evaluation, we tested for the first time a new, extraction-free, protocol with the Allplex SARS-CoV-2 Assay RT-qPCR kit on a Nimbus platform. Ninety-one samples, of which 71 previously tested positive with RT-qPCR with extraction were immediately analyzed without extraction, using only a dilution and thermal shock protocol. The positive and negative percentage agreements were respectively 97.2% (95% confidence interval [CI]: 0.90–0.99) and 95.0% (95% CI: 0.76–0.99). The two false negatives observed were very weakly positive with the comparison method. Moderate variations in Ct of the targeted genes were observed (median ± 95% CI): E gene, +2.49 ± 0.44; N gene, +0.98 ± 0.54; RdRP/S genes, +2.64 ± 0.48. On the other hand, the number of tests performed within 24 h raised from 86.4% to 97.8%, the turn-around time decreased from 19:18 to 09:03 ($p < .0001$), and the number of tests that can be performed per day doubled since this technique was introduced routinely in our laboratory.

KEYWORDS
COVID-19, extraction free, RT-qPCR, SARS-CoV-2

1 | INTRODUCTION

Since it was first described in December 2019, the COVID-19 pandemic has become alarming. In the second half of 2020, in many European countries, a second wave emerged, with a far greater impact than the first wave.[1] At the same time, in Belgium the number of screening indications has also increased sharply: screening before a trip, on a red zone return, or after contact with a confirmed positive patient. The number of people to be tested, either symptomatic or asymptomatic, exploded saturating the laboratories carrying out these molecular analyses. The production capacity can be limited either by shortages of reagents, disposables, instrument saturation, or lack of qualified staff. To save reagents and/or increase testing capacities, various strategies have been developed such as sample pooling, parallel acquisition of new molecular biology techniques, and/or extraction-free SARS -CoV-2 detection.[2–11]

In front of a dramatic health situation with a new, unprecedented, epidemic peak in Europe and a positivity rate of 16.4% on November 22, 2020, in Belgium,[12] the SARS-CoV-2 extraction-free strategy seemed the best option to shorten the turn-around-time of the SARS-CoV-2 RT-qPCR tests and to increase the number of potential runs, paving the way to meeting the ever-increasing demand. Usually, SARS-CoV-2 RT-qPCR tests require a viral RNA extraction, if present, from human cells before amplification. This step can take an additional 3 to 4 h to the 1 to 2 h required for amplification as such. Thus, a complete extraction-amplification platform is capable of assuming a maximum of 3 runs per day, unless night activity is possible in laboratories. However, some

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commercial assays can reduce the extraction time by half by using microplates prefilled with dilution buffers.

In our laboratory, we developed and implemented an extraction-free protocol turning a Nimbus extraction platform into a sample dilution preparation instrument. We analyzed the analytical performances of such a protocol with this widely used instrument or it's equivalent the Starlet detailing the time savings over a period of clinical routine at the height of the epidemic.

2 | MATERIAL AND METHODS

2.1 | Settings

The evaluation was conducted at Iris Hospitals South (HIS-IZZ, Brussels, Belgium), a 4-hospital public network of 550 beds including three laboratory sites. Molecular analyses are carried out in the central laboratory (Lab 1), open 24/7. The other two sites have a local laboratory (Lab 2 and Lab 3) open only on weekdays between 7 am and 5 pm. During the opening hours of these satellite laboratories, the analyses are encoded in the Laboratory Information System (LIS) in situ and the samples reach the main laboratory via regular, once-per-hour, shuttles. Outside these hours, the analyses are encoded in the main laboratory and the samples are delivered by special shuttles upon request.

2.2 | Samples

All samples were fresh nasopharyngeal swabs which were part of routine diagnostic. No freezing-thawing steps were required. A panel of positive samples with various cycle thresholds (Ct) and negative samples were selected for protocol validation. Nasopharyngeal samples for the diagnosis of COVID-19 were taken from UTM-RT swabs (Copan spa, Brescia, IT) or from Vacuette Virus Stabilization Tube (Greiner Bio-One International GmbH, Kremsmünster, Austria).

2.3 | Extraction protocol

Routine extraction protocol (REp) was performed using the STARMag Viral DNA/RNA 200 C Kit (Seegene Technologies) with a Nimbus extraction platform (Seegene Technologies) and the Allplex SARS-CoV-2 Assay RT-qPCR kit (Seegene Technologies) according to the manufacturer's instructions.

2.4 | Extraction-free protocol

The extraction-free protocol (EFp) was performed on the same extraction platform, turning it into a sample and reagent dispenser. Different analytical conditions were previously tested (not published) and those with the best performances were included in the protocol. The clinical samples (15 μL) were diluted (1:4) in 45 μL of RNase-free water into a microwell PCR-plate. Virus inactivation and cell lysis were performed by rapidly heating the plate to 95°C for 5 min. A thermal shock was then applied by cooling the plate to 4°C for 10 min at least. These steps precede the distribution of the mastermix (5 μL of MOM; 4 μL of RNase-free water; 1 μL of internal control; 5 μL of EM8) to which 5 μL of sample is added for RT-qPCR.

2.5 | Amplification

The cDNA synthesis and amplification were performed with a CFX96 C1000 thermal cycler (Bio-Rad Laboratories). The fluorophores used with the Allplex SARS-CoV-2 Assay RT-qPCR kit are FAM, Cal Red 610, Quasar 670 and HEX for detecting E-gene, RdRP gene/S gene, N gene, and the internal control respectively. Results interpretation and Ct calculation were performed with Seegene SARS-CoV-2 Viewer software version 3.19.003.010 (Seegene Technologies). Targets detected with a Ct less than 40 were considered positive. A sample is considered positive if at least one of the targets sought is positive.

2.6 | Calculation of the turn-around time

The turn-around time (TAT) was calculated on the basis of data extracted from the LIS and is based on the elapsed time between the analysis request encoding and the result of technical validation. To compare the TAT of the REp and EFp, we considered two 2-week periods during which each protocol was used solely for period 1, before the implementation of EFp, and period 2 after implementation of EFp. These two periods were separated by 1 week during which the development of the EFp was carried out.

3 | RESULTS

Ninety-one selected samples tested with the REp including 71 positive and 20 negative were retested without extraction immediately after the result was known. The median global Ct (95% CI) was 26.32 (24.69–28.78) and the global dispersion of Ct was 15.5% < 20 cycles, 23.9% between 20 and 25 cycles, 50.7% between 30 and 35 cycles, 9.9% > 35 cycles. The positive and negative percentage agreements were respectively 97.2% (95% CI: 0.90–0.99) and 95.0% (95% CI: 0.76–0.99). The two samples positive with REp but negative with EFp had only one positive target (RdRP/S genes and E gene, respectively) with a Ct value above 39 but below 40. The only positive sample with EFp that was negative with REp showed detection of all the targets with Ct values under 28, but could not be tested with an additional method because of volume constraints. The Ct differences were examined for the different genes and are summarized in Table 1. An analysis of the differences for each Ct gene by the Bland–Altman method did not show a relation between the differences in Ct and the

TABLE 1 Comparison of the differences in Ct with and without extraction

Gene	Linearity	Coefficient of determination	Differences in Ct (median ± 95% CI; min; max)	*p* value (Wilcoxon)
E gene	*y* = 1.0751x + 0.0067	0.9937	+ 2.49 ± 0.44; −3.29; + 5.64	< .0001
N gene	*y* = 1.0146x + 0.0076	0.9902	+ 0.98 ± 0.54; −7.06; + 5.15	.036
RdRP/S genes	*y* = 1.0762x + 0.0095	0.9924	+ 2.64 ± 0.48; −3.17; + 8.01	< .0001

Ct value of the comparison method (REp). The positivity of the RdRP/S, E, and N genes appeared earlier with EFp in 15.9%, 18.8%, and 36.2%, respectively. One gene detected with REp was not detected with EFp in four cases while two genes were not detected in 1 case.

The period 1 using REp-only ran from September 26 to October 11, 2020, including 3077 samples, 21.9% of which were positive. The distribution of samples from Labs 1, 2, and 3 was 57.5%, 25.2%, and 17.3%, respectively. The period 2 using the EFp only ran from October 19 to November 1, 2020, with 3690 samples, of which 45.3% were positive. The sample distribution from Labs 1, 2, and 3 was 53.3%, 25.3%, and 21.4%, respectively. The median TAT during period 1 was 19 h and 18 min (± 95% CI: 0.26 h) versus 9 h and 3 min (± 95% CI: 0.22 h) for period 2 ($p < .0001$, Mann–Whitney *U*-test). The number of tests performed within 24 h raised from 86.4% to 97.8%. Figure 1 emphasizes the differences in TAT between methods.

4 | DISCUSSION

Various studies have already focused on extraction-free protocols.[3–10] Lübke et al.[3] showed with their protocol without extraction using the PrimeDirect TM Probe RT-qPCR Mix (TaKara) that the SARS-CoV-2 detection was 82.4% with 16/91 negative samples characterized by low viral loads (Ct > 35). Barza et al, with their method using the ChromaCode HDPCR TM SARS-CoV-2 Research

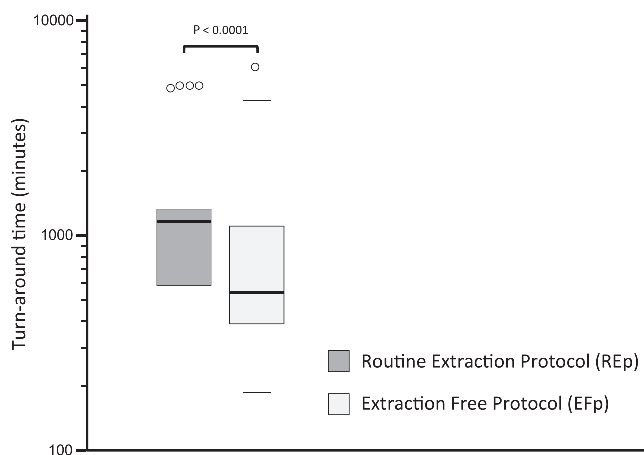

FIGURE 1 Turn-around time with and without extraction using Allplex SARS-CoV-2 assay RTq-PCR kit

Use Only correctly identified 94% of the samples (81/86) with the same observations concerning the negative returned samples (Ct > 35).[8] The extraction-free strategy was also investigated with three other commercial kits showing 51% (37/73) positive detection with SARS-CoV-2 RdRp plus EAV control (Roche), 62% (48/77) with real-time fluorescent RT-qPCR kit for detecting 2019-nCoV (BGI), and 45% (41/74) for Detection kit for 2019-nCoV RNA (Sun Yat-sen University).[9] The Hasan et al.[10] approach based on incubation of 132 nasopharyngeal specimens at 65°C for 10 min along with the use of TaqPath TM1-Step RT-qPCR Master Mix showed a positive percentage agreement of 95%. The addition of proteinase K to heat treatment at 98°C for 5 min delivered a significantly higher positive rate (80%) than those of heat only (58%; $p = .01$).[7] Only one other study showed that direct RT-qPCR without RNA extraction was possible using Seegene if samples are collected in UTM ($n = 70$) and stored at −80°C. However, no sample with a Ct > 35.6 was included in their study.[13]

The protocol proposed in our study adds to the inactivation step of SARS-CoV-2, a thermal shock by cooling the plate to 4°C for 10 min. With the exception of the manual transport of the plate to the heater and to the fridge, the protocol is fully automated. This increases performance, especially for samples with Ct > 35. With this new EFp, the clinical performances are excellent: the positive and negative percentage agreements were, respectively, 97.2% (95% CI: 0.90–0.99) and 95.0% (95% CI: 0.76–0.99). The EFp missed two positives samples previously tested with the REp. However, these two samples each showed only one target detected whose curve came out very late with a Ct value above 39. This is in agreement with other studies relativizing the nondetection of samples with Ct > 35, for which infectivity would be lower.[14–18] On the other hand, the EFp-positive discordant sample had relatively low Ct for all genes. Unfortunately, a false negative with REp could not be determined because the sample could not be retested by a third method due to insufficient quantity. The difference in Ct values between the methods was moderate and did not affect the final results, although the paired analysis showed a statistically significant difference ($p < .05$). Despite the sample dilution, the difference in Ct was not always at the expense of the EFp. In 16% to 36% of positive cases, Ct was even earlier without extraction. In addition, we did not observe a proportional relationship between the Ct difference and the expected Ct values.

To our knowledge, this is also the first study that compared the two techniques (REp vs. EFp) in parallel without having to store the

samples at −80°C. Storage conditions can deteriorate the fragile RNA of SARS-CoV-2 and generate false negative clinical results.[3] The pandemic having impacted the usual distribution chains of PCR reagents and sampling material, such as swabs, laboratories were forced to rely on several suppliers. Therefore, we incorporated UTM and VST media and swabs into the EFp validation.

The two periods studied comparing the TATs were similar in terms of duration, number, and sample type as well as the distribution between our three sites. On the other hand, the rate of positivity was higher in the second period and explained by the epidemic evolution in Belgium during the second wave.[12] The response time has been significantly improved with the EFp, as expected, due to the bypass of the extraction step, which is the most time-limiting one. The Belgian Institute of Public Health, Sciensano, encourages laboratories carrying out SARS-CoV-2 molecular testing to return the results within 24 h. In the first period, 86.4% of the results were answered within 24 h, compared to 97.8% in the second period. In addition, the number of possible runs per 8-h workday goes from 2 with the REp to 4 with the EFp or 288 samples per day instead of 144.

5 | CONCLUSION

The second wave of the COVID-19 epidemic led to a very high demand in terms of molecular analyzes of SARS-CoV-2 from clinical biology laboratories. The implementation of a new, extraction-free, protocol-based solely on thermal shock and sample dilution allowed a median gain of 10 h in the rendering of results. This protocol also helped to better meet national requirements and to communicate almost all results within 24 h.

In addition, it made it possible to double the number of samples tested per day while keeping the same working hours. The test performances being excellent, we decided to implement this extraction-free method in routine immediately after this evaluation.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

Laurent Blairon and Marie Tré-Hardy conceptualized and designed the study. Sébastien Piteüs analyzed the samples. Laurent Blairon and Marie Tré-Hardy analyzed and interpreted the data. Laurent Blairon and Marie Tré-Hardy drafted the manuscript. Laurent Blairon performed the statistical analysis. Laurent Blairon, Sébastien Piteüs, Ingrid Beukinga, and Marie Tré-Hardy critically revised the manuscript. Laurent Blairon and Marie Tré-Hardy supervised the study.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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