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# Evaluation of extraction-free RT-PCR methods for faster and cheaper detection of SARS-CoV-2 using two commercial systems

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ABSTRACT

*Objective:* When using high-throughput batched diagnostic platforms based on RT-PCR for SARS-CoV-2 detection, avoidance of the conventional nucleic acid extraction step can help to reduce the turnaround time and increase processivity. This approach can also spare reagents and plasticware, which have experienced a shortage during the initial waves of the pandemic, reducing the overall testing costs.
*Methods:* This study evaluated the performance of extraction-free protocols based on simple dilution of the specimen in sterile RNAse free water (with or without a heating step) in comparison to standard RNA extraction protocols, using two commercial kits for molecular detection of SARS-CoV-2 (Allplex™ SARS-CoV-2 assay and Allplex™ SARS-CoV-2/FluA/FluB/RSV assay) in nasopharyngeal swabs (NPS).
*Results:* Compared with conventional protocols, extraction-free protocols based on sample dilution without a heating step exhibited a lower analytical sensitivity: 74.0% and 82.1% with the Allplex™ SARS-CoV-2 assay (tested with 139 NPS samples) and the Allplex™ SARS-CoV-2/FluA/FluB/RSV assay (tested with 69 NPS samples), with a mean increase of Ct values of +2.04 and +1.32, respectively. Most false negative results were observed with sampled low viral load. Including a step of heat exposure did not improve but actually decreased the analytical sensitivity of the assay.
*Conclusions:* Results confirmed that extraction-free protocols could be a faster and cheaper approach to SARS-CoV-2 detection in NPS samples, which could improve processivity of diagnostic platforms.


## Introduction

Real-time reverse transcriptase-polymerase chain reaction (rRT-PCR) analysis of nasopharyngeal swabs (NPS) or other respiratory specimens represents the reference method for SARS-CoV-2 infection diagnosis (WHO, 2020).

Analytical platforms which can process large batches of specimens allow high-throughput testing but require relatively long turn-around times (TAT), due to the need for an RNA extraction step followed by PCR set-up and finally thermal cycling.

Several studies have recently evaluated the possibility of SARS-CoV-2 RNA detection without a conventional RNA extraction step, to reduce TAT and spare extraction reagents and plasticware, which during the pandemic waves have experienced a serious shortage (Brown *et al.*, 2020; Bruce *et al.*, 2020; Merindol *et al.*, 2020). In particular, a heating step for inhibitors inactivation and RNA release, performed before rRT-PCR in place of conventional extraction, was proposed as a possible alternative for faster SARS-CoV-2 detection in nasopharyngeal swabs (Barza *et al.*, 2020; Lübke *et al.*, 2020).

The aim of this study was to evaluate the performance of rapid extraction-free protocols using two commercial rRT-PCR assays.

## Materials and Methods

The material analysed with extraction-free methods was represented by anonymized residual samples of NPS in UTM® medium (Copan, Italy) submitted to the Microbiology and Virology Unit of Florence Careggi University Hospital (Florence, Italy) for SARS-CoV-2 detection in the period August - November 2020. The samples were selected among those already processed with the corresponding conventional method within 24 hours, and were always stored at 4°C.

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**Table 1**
Results obtained with the extraction-free methods in comparison with the conventional method.

| Assay | Method | Positives | Negatives | SE % (95% CI) | SP % (95% CI) | PPV % (95% CI) | NPV % (95% CI) | TOTAL |
|---|---|---|---|---|---|---|---|---|
| ALLPLEX-COV | STARMAG | 96 | 43 | - | - | – | – | 139 |
| | EX-FREE | 71 | 68 | 74.0 (64.6-81.9) | 100 (94.4-100) | 100 (96.5-100) | 63.2 (51.4-74.0) | |
| | EX-FREE-HEAT | 55 | 84 | 57.3 (47.3-66.8) | 100 (94.4-100) | 100 (95.6-100) | 51.2 (40.6-61.7) | |
| ALLPLEX-COV-FLU | STARMAG | 39 | 30 | - | - | - | - | 69 |
| | EX-FREE | 32 | 37 | 82.1 (68.0-91.6) | 100 (92.0-100) | 100 (92.5-100.0) | 81.1 (66.4-91.1) | |

SE: analytical sensitivity; SP: specificity; PPV: positive predictive value; NPV: negative predictive value.

A first group of 139 samples was evaluated using the Allplex™ SARS-CoV-2 assay (ALLPLEX-COV, Seegene Inc, South Korea) with three different methods: i) RNA extraction with the STARMag 96 × 4 Universal Cartridge Kit (Seegene Inc, South Korea) followed by PCR set-up and cycling (STARMAG protocol); ii) direct PCR set-up after dilution of the sample (1:4) in sterile RNase-free water (EX-FREE protocol); iii) direct PCR set-up after dilution of the sample (1:4) in sterile RNase-free water and heating at 98°C for 3 minutes (EX-FREE-HEAT protocol). The ALLPLEX-COV assay consists of a multiplex rRT-PCR able to simultaneously detect four viral targets including the envelope (*E*) gene, the RNA-dependent RNA polymerase (*RdRP*) gene, the spike (*S*) gene, the nucleocapsid (*N*) gene, and an exogenous RNA-based internal control (IC). According to the manufacturer's indications, samples were considered positive when a signal was detected at cycle threshold (Ct) <40 for any target gene, negative if only the IC was amplified, and invalid if the IC was not amplified. The reverse transcriptase reaction was carried out at 50°C for 20 minutes, followed by a step at 95°C for 15 minutes. The PCR reaction consisted of 45 cycles of 10 seconds at 95°C, 15 seconds at 60°C and 10 seconds at 72°C.

A second group of 69 samples was evaluated using the Allplex™ SARS-CoV-2/FluA/FluB/RSV assay (ALLPLEX-COV-FLU) with the STARMAG protocol and the EX-FREE protocol. The ALLPLEX-COV-FLU assay performs a multiplex rRT-PCR able to simultaneously detect three SARS-CoV-2 viral targets, including the *E, RdRP* and *N* genes, an exogenous RNA-based IC, and an endogenous DNA-based IC. Samples were considered positive when a signal was detected at Ct <40 for any target gene, negative if only the ICs were amplified, or invalid in the absence of amplification of the endogenous and/or exogenous IC. The reverse transcriptase reaction was carried out at 50°C for 20 minutes, followed by a step at 95°C for 15 minutes. The PCR reaction consisted of 42 cycles of 10 seconds at 95°C, 15 seconds at 60°C, and 10 seconds at 72°C.

All samples were processed using a Hamilton Microlab NIMBUS automated extraction and PCR setup system (Hamilton Company, USA), and rRT-PCR was performed with a CFX96 thermal cycler (BioRad, USA). rRT-PCR results were interpreted with the SARS-CoV-2 viewer software according to manufacturer's instructions.

Graphical projections and statistical analysis (*p* value, *r* squared) were performed using GraphPad Prism 7.0 (GraphPad Software, Inc., San Diego, CA). Percentages of analytical sensitivity, specificity, positive predictive value and negative predictive value with their 95% confidence intervals (CI) were calculated using the "Jeffreys" method (http://www.ausvet.com.au).

## Results

### *Comparison of results obtained with the ALLPLEX-COV assay using conventional extraction vs. two extraction-free protocols*

Overall, 139 samples were included in this evaluation, including 96 positives and 43 negatives with the standard methodology using the extraction step recommended by the manufacturer (STARMAG protocol), and considered as true positives (TP) and true negatives (TN), respectively. The mean cycle threshold (Ct) values with the TP samples were 30.03, 30.23 and 29.72 for the *E, RdRP/S* and *N* genes (ranges 15.85-38.53, 14.9-39.64, 14.6-38.88), respectively.

Of the TP, 71/96 (74.0%) and 55/96 (57.3%) were positive with the EX-FREE and the EX-FREE-HEAT protocols, respectively, while no TN sample was called positive by the two extraction-free protocols (Table 1).

The false negative (FN) results yielded by the extraction-free protocols were observed with positive samples that exhibited high Ct values with the STARMAG protocol: mean 36.29 (range 33.57-38.53), 36.60 (range 31.93-39.05) and 35.95 (range 30.94-38.88) for the *E, RdRP/S* and *N* genes respectively with the EX-FREE-HEAT protocol; mean 36.06 (range 34.15-38.53), 36.48 (range 32.65-39.05) and 36.24 (range 32.81-38.88) for the *E, RdRP/S* and *N* genes, respectively, with the EX-FREE protocol (Figure 1).

Comparison of the Ct values exhibited by samples positive for each of the two extraction-free protocols vs. the standard protocol revealed an average ΔCt of +1.59, +1.86 and +2.69 ($p<0.001$) for the *E, RdRP*/*S* and *N* genes, respectively, with EX-FREE protocol, and an average ΔCt of +3.98, +4.02, +4.49 ($p<0.001$) for the *E, RdRP*/*S* and *N* genes, respectively, with the EX-FREE-HEAT protocol (Figure 1).

Altogether, these results showed that the extraction-free protocols exhibited an overall lower analytical sensitivity than the conventional protocol, and that the EX-FREE protocol exhibited a higher analytical sensitivity than the EX-FREE-HEAT protocol (Table 1). In fact, samples called as FN with the EX-FREE protocol always exhibited Ct values >32 with the conventional STARMAG protocol.

### *Comparison of results obtained with the ALLPLEX-COV-FLU assay using conventional extraction vs. an extraction-free protocol*

Overall, 69 samples were included in this part of the study. Of these, 39 were TP and 30 TN (Table 1). The mean Ct values among the 39 TP were 24.13, 22.92 and 23.53 for *S, R* and *N* genes (ranges 12.5-38.6, 12.7-39.2, 13.61-36.34), respectively. Considering the higher analytical sensitivity obtained with ALLPLEX-COV and the EX-FREE protocol, only the latter extraction-free protocol was tested in comparison with the conventional protocol.

Of the TP, 32/39 (82.1%) were positive with the EX-FREE protocol, while no TN sample was called positive by the EX-FREE protocol.

The FN results yielded by the EX-FREE protocol were mostly observed with positive samples that exhibited high Ct values (>34) with the STARMAG protocol, except for one sample which showed lower Ct values (20.03, 20.1 and 19.82 for the *S, R* and *N* gene, respectively). The means and ranges of Ct values observed with positive samples reported as negative by the EX-FREE protocols were 33.99 (range 20.03-38.6), 28.58 (range 20.1-37.1), and 32.76 (range 19.82-36.34) for the *S, R* and *N* gene targets, respectively (Figure 2).

Comparison of the Ct values exhibited by samples positive with the EX-FREE vs. STARMAG protocols revealed an average ΔCt of

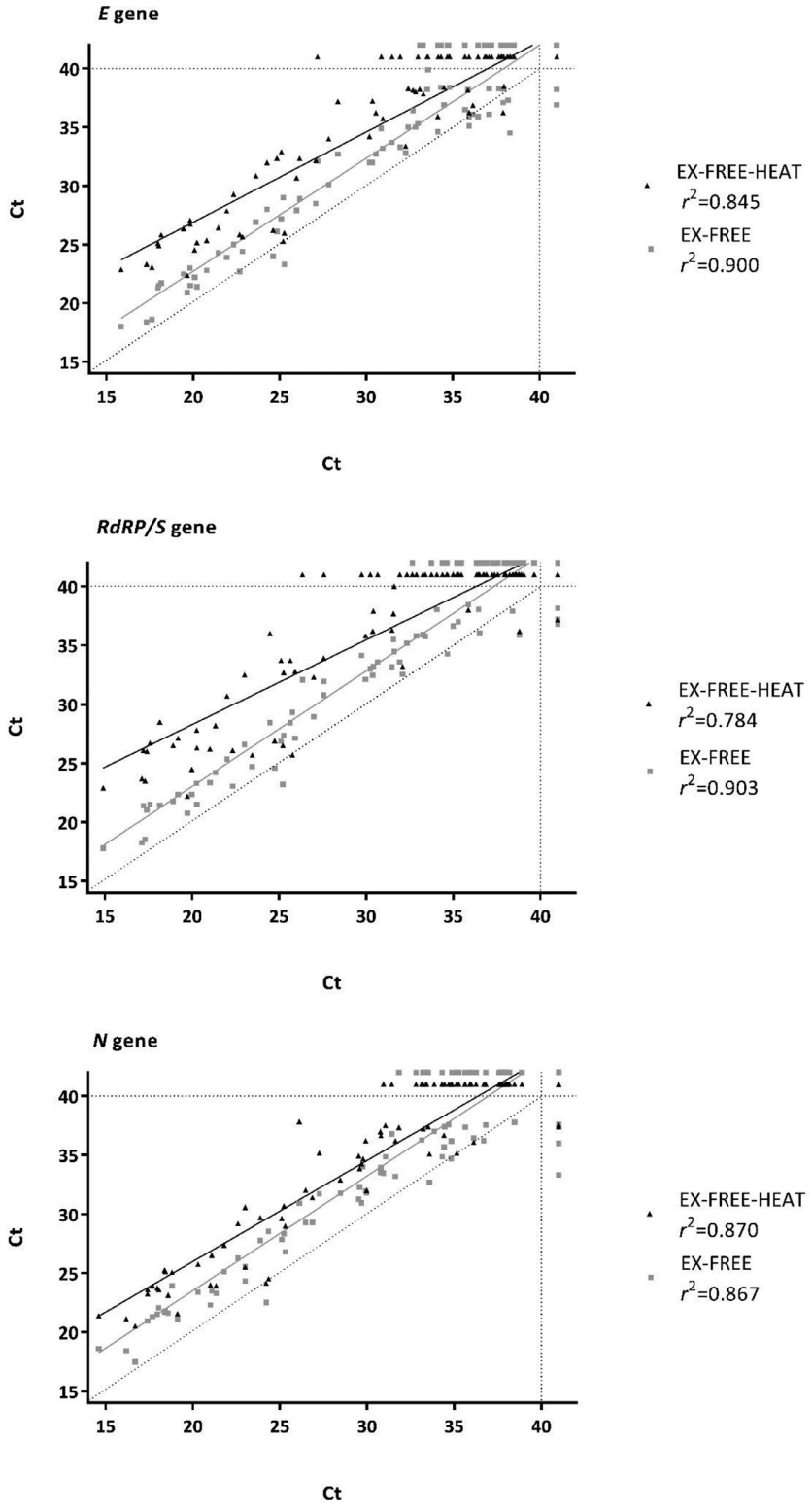


**Figure 1.** Comparison of each gene Ct with positive samples obtained with the STARMAG protocol (on *x* axis), the EX-FREE protocol and the EX-FREE-HEAT protocol using the ALLPLEX-COV kit. Negative targets were plotted as Ct >40.

+1.29, +1.63 and +1.06 ($p<0.001$) for *S*, *R* and *N* genes, respectively.

These results showed that also with the ALLPLEX-COV-FLU the EX-FREE protocol exhibited an overall lower analytical sensitivity than the STARMAG protocol.

Endogenous IC analysis also showed an increase of average Ct values with the EX-FREE protocol ($p<0.001$) with both positive (+2.84) and negative (+4.06) samples (Figure 3), that was higher than the ΔCt value observed with SARS-CoV-2 targets ($p<0.05$). This might be explained by a higher difficulty to release human cellular material by the EX-FREE protocol.

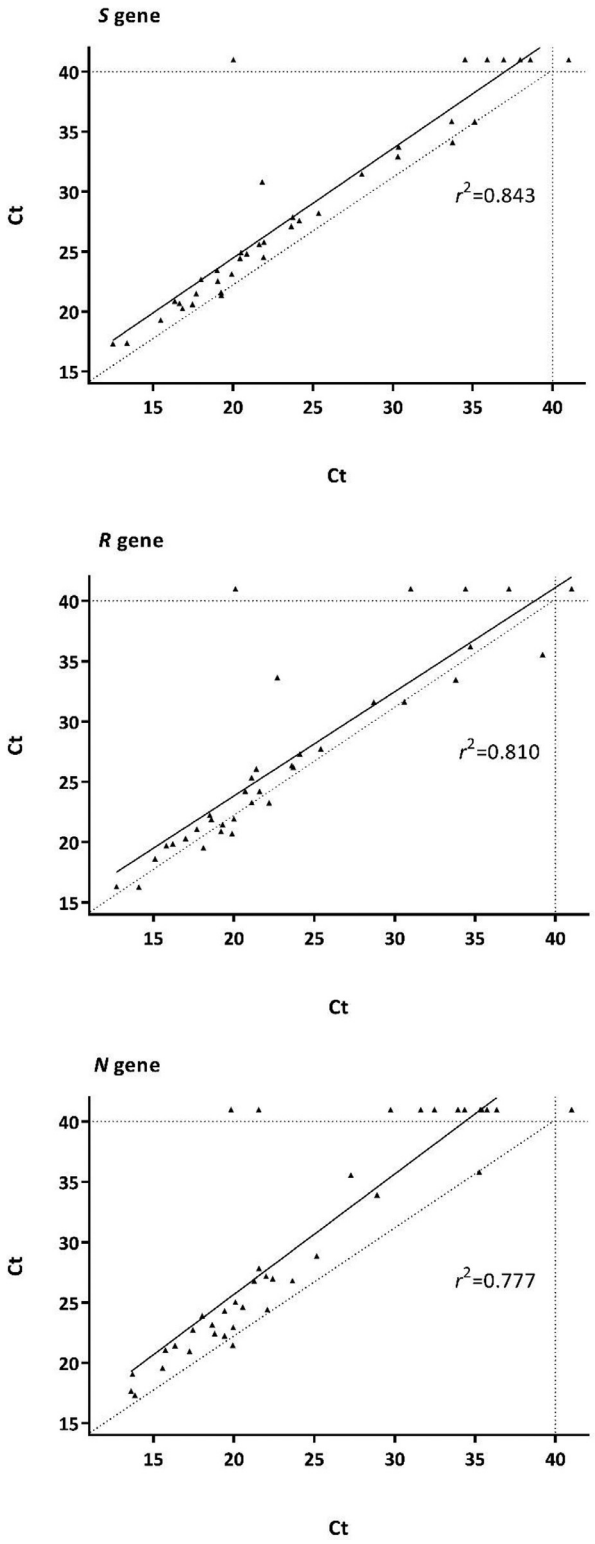


**Figure 2.** Comparison of each target gene Ct with positive samples obtained with the STARMAG protocol (on *x* axis) and the EX-FREE protocol using the ALLPLEX-COV-FLU kit. Negative targets were plotted as Ct >40.

## Discussion

During the initial waves of the COVID-19 pandemic, a rapidly increasing demand for molecular testing has been experienced on a global scale, while diagnostic laboratories have often experienced difficulties in complying with the testing workload due to shortages of reagents, plasticware, diagnostic platforms and manpower (Barra *et al.*, 2021).

Under these circumstances, the possibility to shorten the analytical workflow by skipping the conventional nucleic acid extraction step, when using high-throughput RT-PCR diagnostic platforms that rely upon separate steps of RNA extraction, PCR set-up and amplification, appeared an appealing alternative to reduce the TAT which could also spare reagents and plasticware (Smyrlaki *et al.*, 2020). Nowadays, the shortage of diagnostics for SARS-CoV-2 detection is no longer a major issue in high-income countries, but it remains an issue in medium- and low-income countries. Moreover, extraction-free methods allow TAT and cost reduction which are of interest for all settings and may be helpful for detection of other viral pathogens.

Results obtained in this study revealed that a simple dilution step of the UTM matrix used for NPS in sterile RNAse-free water could be successfully used in place of the conventional RNA extraction step with two different commercial rRT-PCR assays. As expected, skipping the conventional nucleic acid extraction protocol (which also results in some concentration of the sample) was associated with some reduction of the analytical sensitivity of the test. However, the reduction was overall moderate, especially with the simplest protocol which did not include a heating step. In fact, the EX-FREE method showed a higher analytical sensitivity and a better concordance with the STARMAG protocol than the EX-FREE-HEAT protocol, when using the ALLPLEX-COV kit.

The relatively large amount of false negatives with positive samples yielding high Ct values in the conventional test revealed a loss of accuracy for extraction-free protocols with samples with a low viral load. However, these cases are known to exhibit lower infectivity (Singanayagam *et al.*, 2020) and are often typical of the late stages of infection, when diagnosis has already been made.

To the best of our knowledge, this is the first study where ALLPLEX-COV and ALLPLEX-COV-FLU assays used with conventional and extraction-free protocols were compared. Previous studies (Ambrosi *et al.*, 2021; Freppel *et al.*, 2020) were carried out with Allplex$^{TM}$ 2019-nCoV assay (Seegene Inc.), which was available before the former systems and did not target the *S* gene nor include an endogenous DNA-based IC.

Previous studies revealed that a thermal lysis step (90°C for 3 min) of 1:5 diluted samples slightly improved the efficiency of SARS-CoV-2 detection (Freppel *et al.*, 2020). Comparable results were obtained with a longer heating step (65°C for 20 min), but without sample dilution and with a different rRT-PCR protocol (Barza *et al.*, 2020). Different sample dilution ratios and heating steps could explain at least in part the variability observed between previous and our data.

A limitation of this study was that, since ALLPLEX-COV and ALLPLEX-COV-FLU were provided at different times, it was impossible to make a comparison between the two systems with the same set of samples.

## Conclusions

The feasibility of extraction-free SARS-CoV-2 RNA detection protocols was evaluated with two high-throughput rRT-PCT assays. Results revealed that, despite some reduction of analytical sensitivity, these protocols could be an acceptable method for SARS-CoV-2 detection in clinical samples in case of shortage of the extraction reagents. Moreover, the use of these extraction-free protocols, which are cheaper and faster than the conventional protocols using a nucleic acid extraction step, could reduce testing costs and increase processivity of the analytical platforms.

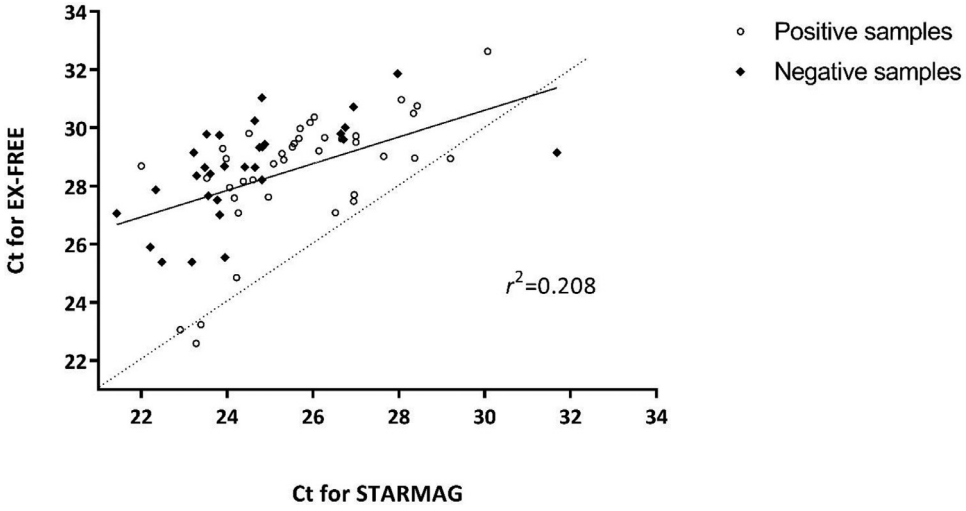


**Figure 3.** Endogenous IC Ct values obtained with ALLPLEX-COV-FLU and the two analytical protocols.

## Declaration of Competing Interest

Dr. Antonelli reports personal fees from Accelerate diagnostics, personal fees from Menarini, personal fees from Seegene, outside the submitted work.

Prof. Rossolini reports grants, personal fees and non-financial support from Accelerate Diagnostics, personal fees from Becton Dickinson, grants and personal fees from bioMeriéux, grants and personal fees from Cepheid, grants and personal fees from Elitech, grants and personal fees from Merck, grants and personal fees from Nordic Pharma, personal fees from Pfizer, grants from Seegene, grants and personal fees from Shionogi, personal fees and other from Venatorx, grants and personal fees from Zambon, personal fees from Roche, personal fees from Thermo Fisher, personal fees and non-financial support from Beckman Coulter, grants, personal fees and non-financial support from Menarini, grants from Arrow, grants from Symcel, personal fees from QPex, grants from DID, grants from Hain Lifescience GmbH, grants from GenePoc, grants from SetLance, grants and personal fees from Angelini, grants from Qvella, grants from Qlinea, personal fees from Qiagen, grants from Biomedical Service, grants from Liofilchem, outside the submitted work.

Dr. Baccani reports other from Diesse-Diagnostica Senese, outside the submitted work.

All other authors have nothing to disclose.

## CRediT authorship contribution statement

**Fabio Morecchiato:** Methodology, Investigation, Data curation, Writing – original draft, Writing – review & editing. **Marco Coppi:** Conceptualization, Methodology, Investigation, Data curation, Writing – original draft, Writing – review & editing, Writing – review & editing. **Ilaria Baccani:** Conceptualization, Methodology, Investigation, Data curation, Writing – original draft, Writing – review & editing. **Niccolò Maggini:** Investigation, Data curation, Writing – review & editing, Supervision. **Nunziata Ciccone:** Data curation, Supervision. **Alberto Antonelli:** Conceptualization, Methodology, Investigation, Data curation, Writing – original draft, Writing – review & editing. **Gian Maria Rossolini:** Conceptualization, Writing – review & editing, Supervision.

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## Ethical approval

Not required.

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