Lab practices that improve coronavirus disease 2019 detection accuracy using real-time PCR

Ghadir Soufan,[1,*] Nour Berro,[1,*] Sahar Nahle,[1,2,*] Malak Darwish,[1] Maysaa Ghaddar,[1] Mohamad Makki,[1] Mahmoud Younes[1] and Fadi Abdel Sater[1,2]

[1]*Medical Research Department, Al Rasoul Al-Aazam Hospital,* [2]*Biochemistry Department, Faculty of Sciences (I), Lebanese University, Beirut, Lebanon*

ABSTRACT

The number of coronavirus disease 2019 (COVID-19) cases significantly increased with the emergence of multiple variants of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). This has led to an ongoing effort focused on developing the diagnostic detection tests. Among the currently available tests, real-time reverse transcriptase PCR (RT-PCR) has been considered as the `golden method' for the detection of SARS-COV-2. However, a significant number of inaccurate (false-negative/false-positive) results have been reported in spite of this method's reliability and effectiveness. These unreliable results may arise because of various issues encountered throughout the entire testing process starting with the sampling phase, going through the PCR process, and ending with the result analysis. This article aims to shed light on the errors that occur during the COVID-19 testing process and suggest ways to overcome them effectively. Accurate testing could be optimized by following the correct swabbing technique, using adequate RT-PCR kits and controls, setting clear lab guidelines, and properly interpreting the results.

Key words: coronavirus disease 2019, false-negative, false-positive, lab procedure, real-time PCR

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What is known about the topic?
- Real-time PCR is the golden method of COVID-19 diagnosis, yet it is prone to giving false-positive or false-negative results.
- SARS-CoV-2 extraction method is a multistep procedure; thus, it is possible to have problems during each step, which affects the quality of the results.
- The detection of the virus occurs using different amplification kits that vary in their sensitivities.

What does this article add?
- This article adds insights and information on the proper way to handle PCR samples in order to improve the accuracy of the results.
- It gives guidance to the technicians, thus reducing any inconsistencies in the results.
- It provides beneficial information about the possible errors that arise during COVID-19 detection and shares tips that help with detecting and overcoming these errors.

Correspondence: Ghadir Soufan, Medical Research Department, Al Rasoul Al-Aazam Hospital, Beirut, LB, Lebanon.
E-mail: Ghadeer.soufan.4@hotmail.com

*G.S., N.B. and S.N. contributed equally to this work.

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Introduction

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) invaded our lives and redefined all our social standards. Since its onset in December 2019, the novel virus has forced countries worldwide into lockdowns and restricted social events.[1] The pandemic has challenged us in a lot of ways, and we have had to adapt by maintaining social distancing and transferring face-to-face public events onto digital online platforms.[2] The coronavirus disease 2019 (COVID-19) also caused significant morbidity and mortality rates among infected patients.[3] Therefore, the global public crisis has resulted in health, economic, and social ramifications.[4]

On 11 March 2020, the WHO declared COVID-19 as a pandemic disease that has affected tens of millions of people all over the world.[5] Most COVID-19-infected patients present with various symptoms including fever, dry cough, fatigue, and dyspnea. Some patients have no symptoms at all.[6] The Centers for Disease Control and

Prevention (CDC) and WHO recommended several precautions to avoid infection by COVID-19 including social distancing, wearing face masks, thoroughly washing hands, and staying away from people displaying flu-like symptoms.[7] The clinical characteristics of COVID-19 can also be associated with other infections that display similar flu-like symptoms,[8] which is why a diagnostic test is essential in accurately identifying possibly contagious positive cases.[9] The accurate identification and quarantine of those cases helps in curbing the spread of the virus among populations.[7]

The different types of tests used for COVID-19 detection include molecular, antigen, and antibody tests.[10] Molecular testing, based on the genetic material of the virus, is known to be the most accurate test.[11] Since December 2019, the PCR, especially real-time reverse transcriptase-PCR (RT-PCR), has been considered a `golden method' for the detection SARS-CoV-2.[12] The early availability of the complete genome of SARS-CoV-2 helped in designing specific primers and probes that target specific SARS-CoV-2-related genes, such as: *RNA-dependent RNA polymerase* (*RdRp*), *envelope* (*E*), *nucleocapsid* (*N*), and *spike proteins* (*S*).[13] Currently, RT-PCR plays an important role in SARS-CoV-2 detection because of its specificity and simplicity[14] especially during the early stages of infection.[15] Although the RT-PCR in SARS-CoV-2 diagnosis is of great significance and benefit, it might sometimes give inaccurate results (false-negative or false-positive results) because of errors committed by technicians, such as mixing up samples or faulty result analysis.[16,17]

In the present article, we aim to critique the COVID-19 testing procedure. We also aim to focus on the errors that our lab has faced during the detection of SARS-CoV-2 by real-time RT-PCR starting from the sampling step, through the process of PCR, to the result analysis step. Additionally, we present different solutions that could help reduce the inconsistencies in the results and improve the laboratory procedures.

Improper swabbing

Respiratory cells containing the novel coronavirus can be obtained through nasopharyngeal swabs, sputum samples, deep tracheal aspirates (DTA), throat swabs, and broncho-alvoelar lavage fluid (BALF).[18] The virus's RNA is detected by using the RT-PCR technique on the respiratory cells collected from the infected patients.[19] One study has suggested that a sputum sample is the most efficient in detecting COVID-19 because of its high viral load.[18] However, we have noticed that if the sputum sample was too viscous, its high viscosity interferes with the extraction of RNA from the cells. That is why we believe that the nasopharyngeal swabbing is the optimal sample collection method for COVID-19 testing. Improper swabbing can lead to 30% false-negative results and to a decrease in the test's sensitivity. Therefore, in order to obtain a reliable result, it is critical to properly execute the swabbing technique.[20]

Patients must sit with their heads in a straight and forward position without movement. The same swab is then inserted straight back into both nasal cavities. The handler must gently roll the swab for a few seconds to absorb the secretions, then gently remove the swab while continuing to rotate it. The swab is then placed in a sterile transport tube containing 2–3 ml of either viral transport medium (VTM), PBS, or sterile saline. The volume in the transport tube should not exceed 2–3 ml so the cells would not be diluted. If the cells in the sample were too diluted, this may lead to a false-negative result.[21]

Mislabeling

Laboratories responsible for PCR testing started facing a heavier workload because of the drastic increase in the positive tested cases and the demand for PCR tests. This increased the risk of errors at the labs.[22]

At our lab, one of those errors was mislabeling. Mislabeling is when one patient's test tube is labeled with another patient's name. In such a case, the patients' results become mixed up. To avoid this error, patients must first loudly state their names while presenting a copy of their identification (ID). Then, the handler must correctly label the tube with the patient's name before placing the respective swab in it and moving on to the next patient. We have also found it best to administer one patient at a time in order to avoid any mix ups.

Possible leakage and poor storage of the samples

After taking the nasopharyngeal swab, it is essential to ensure the proper sealing of the sample to avoid leakage. The sample should also be placed in a double zipper bag to prevent any cross-contamination with other samples. All samples must be stored at low temperatures (2–8°) until they reach the molecular lab.[21]

In case of a leakage, some of the samples may contaminate the handler's gloves and the surrounding working area. At our lab, this has sometimes led to cross-contamination between the samples causing false-positive results. For this reason, when a leakage is noticed, it is preferable to handle the leaking sample away from the other samples, cleaning the surrounding area with ethanol, and changing the handler's gloves before touching the other samples.

Extraction issues

In the case of automated extraction

Sample loading

During automated extraction, there is a risk of contamination in the wells while loading the samples since the 96-deep well plate has an exposed surface. During our runs, we have noticed that it is possible for a drop hanging on the outer edge of the tube of a positive sample to fall into the well of a negative sample. The negative well, thus becomes contaminated and results in a false-positive. We have handled this risk by opening the samples as far away as possible from the deep well plate and removing any excess droplets present on the outside of the tube using a tissue covered with ethanol.

Accidental pooling and cross contamination

Another human error we have faced is the accidental pooling of one sample in the same well of another sample. This leaves one well without a sample and another well with two samples on top of each other. This may lead to a false-negative result coming from the well without a sample. It may also lead to a false-positive result; in case a positive sample was pooled on top of a negative one.

We were able to detect this error during result analysis when one of the wells appeared without an endogenous internal control. We were also able to notice the accidental pooling of two samples on top of each other as we used deep well plates with transparent bases. After loading each column with samples, we check the base of the plate to see if the volume in all the wells is the same. If one well has less volume than the other wells and another well has more volume, we can predict that these two samples were loaded on top of each other in the same well.

Cross-contamination is also possible if the tip, which was used to load a positive sample accidentally touched a well designated for another sample. Thus, it would be beneficial to leave out two empty wells in the 96-deep well plate for the reloading of the samples corresponding to the contaminated wells.

Orientation of deep wells on the automated extractor

Automated extraction machines, such as the Kingfisher Flex by Thermo Scientific, have a specific orientation in which the deep well plates have to be placed. If a plate was placed in the opposite direction in the machine, all the samples' positions would be reversed (well 12H would be in the position of well 1A, for example). In this case, the patients might receive the wrong result (patient corresponding to well 1A would obtain the result corresponding to well 12H). We start our runs by first clearly marking the front of the plates. This has helped us in avoiding any confusion while loading the deep well plates onto the machine.

RNA loading

Another human error we have encountered was during the transfer of RNA from the deep well plate into their corresponding wells in the 96 PCR amplification plate.

At one time, a colleague transferred the RNA of one sample into the corresponding amplification well of another sample. This led to a false-positive result when the RNA of the positive sample was pooled onto the RNA of a negative sample. In a different instance, one co-worker skipped loading the RNA of a sample into its corresponding amplification well. This error was detected by the absence of an internal control in that well during result analysis.

We have organized our work in such a way to avoid these errors. While loading RNA into a 96 PCR plate, we use a 96-tip box. Every tip in the box corresponds to the well that needs to be loaded. This has helped us keep track of the wells that have been loaded and their location in the plate. Also, we started using transparent 96 PCR amplification plates. After loading each column in the plate, we check if the volumes in all the wells are equal by looking at the bottom of the plate. In addition, we have turned to using a multi-channel pipette to load the RNA, which helped us avoid these errors and save time.

Another error was accidentally loading the positive control in the well right above its designated well giving its sample a false-positive result. We detected this mistake during result analysis when we noticed that the positive control was missing (no curves were displayed indicating no amplification).

In the case of manual extraction

Sample loading

During manual extraction, each labelled sample needs to be loaded into its respective labelled Eppendorf tube. One error our team has made during this step was accidently loading a labelled sample (carrying the number 6) into a differently labelled Eppendorf tube (carrying the number 9), thus mixing up the samples. We have also once overlooked a labelled Eppendorf tube without loading any sample into it, which resulted in a false-negative result when the internal control was exogenous. Moreover, we once loaded a sample into an empty Eppendorf tube not containing any lysis buffer. We ended up repeating the sample's extraction after the result analysis showed no amplification of an internal

control. These problems can be solved by careful labeling, double checking the prepared spin columns and Eppendorf tubes, and by using the appropriate extraction controls that allow the detection of such errors during result analysis.

Mixing up the used solutions

During manual extraction, we first add the sample to the lysis buffer. After that, ethanol is added to the sample-lysis mix. In our lab, ethanol, which is transparent, is often made into aliquots in falcons. We also aliquot distilled water into similar falcons. This caused a mix-up as the falcons are identical and both the ethanol and distilled water are transparent. During one of the runs, a colleague added distilled water instead of ethanol to the sample-lysis mix. This error inhibited the RNA extraction process. To avoid this, either properly and clearly label all the falcons or use falcons with different colored caps for different solutions.

Cross-contamination

Some manual extraction kits, such as Qiagen, provide large quantities of their solutions (wash1, wash2, elution) in the same container for continuous usage. Inserting a pipette directly into that container may lead to its contamination if the pipette was not thoroughly cleaned and sterilized after each use. Preparing aliquots of the solutions minimizes the risk of contamination in such cases.

Mixing up samples

Another problem we have faced during manual extraction was during the binding step. A colleague transferred the sample-ethanol-lysis mix from the Eppendorf tube labelled with the number 15 into a spin column labelled with the number 51, a transposition error. Also, during the elution step, the spin column corresponding to a specific sample (number 1) was placed in the elution Eppendorf tube corresponding to a different sample (number 16). Eventually sample number 1 was loaded into the amplification mix as sample number 16. We dealt with that risk by always lining up all of our Eppendorf tubes or spin columns in increasing order from left to right. We also started double checking the labels on each tube or column before every step.

Errors during the preparation of amplification plates

Master mix preparation

We have faced several issues during the preparation of the master mix. The enzyme is usually required in very small quantities in several amplification kits, such as the Life River kit, Maccura kit, and ANDiS kit. When the tip was not well secured onto the pipette, the required enzyme quantity could not be drawn and its absence was difficult to detect. This rendered the amplification mix ineffective and led to the absence of any amplification of RNA.

We had to handle two other errors during master mix preparation. The first was when we forgot to add one of the components required to complete the master mix. The other was when we added the right component but in the wrong amount as the pipette measurement was not checked first. Both resulted in an ineffective master mix. An ineffective master mix could be detected by the absence of amplification of the positive control.

Moreover, a lot of kits use transparent components, like the Maccura kit. These components could be confused with other transparent liquids, such as water if not properly labeled and separated.

Such problems can be avoided by proper work space organization and clear labelling. It is also beneficial to have two different people working throughout the process; one handling the RNA extraction and the other handling the master mix preparation.

Sealing the 96 well amplification plate

During the PCR process, the samples are exposed to high temperatures. So, in the case the wells that contain RNA are not sealed adequately, the well contents evaporate.[12] Hence, improper sealing of a loaded amplification plate can lead to cross contamination between samples. Our example of improper sealing was when the seal was not evenly distributed around the edges of the amplification plate. Also, it was not pressed down properly allowing the air to seep in and lift it up.

And when one of the evaporated samples was positive, it contaminated the entire BioRad PCR machine that we use. We started seeing unspecific curves in the results while using the machine later and had to decontaminate it. To deal with that risk, we made sure that we properly sealed the amplification plate and we checked the edges of the seal before placing it into the PCR machine. Another error we faced was when we placed the amplification plate into the QuantStudio PCR machine in the inverse position. Well 1A should have been placed towards the upper left corner, instead it was placed towards the lower right corner (the plate's orientation was inversed). Thus, each result was ascribed to the wrong well during analysis. So, it is very important to secure the plate in its place in the right position before starting the run.

Issues faced during RT-PCR result analysis

Analysis of the internal control

One of the first things we look at while analyzing the RT-PCR results is the internal control. An internal control assures that both the extraction and amplification processes went well.

Some amplification kits provide an exogenous internal control, such as LifeRiver, TaqPath, ANDiS, and Maccura kit. An exogenous internal control is added to each sample before the beginning of the extraction process and its amplification indicates successful extraction and amplification.

Other kits provide an endogenous internal control, such as Bosphore, Virucell, Sinmurvv, and BGI. An endogenous internal control is found within the human respiratory cells. So, the amplification of an endogenous internal control not only indicates successful extraction and amplification but also ensures that the sample was properly collected and there were enough cells for extraction.

Therefore, verifying the amplification of internal controls is essential before validating the negative results. For example, while analyzing the RT-PCR results, a sample appeared negative as it did not show any amplification in the virus's targeted genes. Yet, upon further analysis, we noticed that there also was not any amplification of the exogenous internal control of the same sample. And at the same time, the positive control was amplified assuring the efficiency of the master mix. We then concluded that the nucleic acid extraction was unsuccessful as the internal control was not extracted. So, we repeated the extraction then amplification of this sample before verifying its result.

One of the issues we faced while analyzing the result of an RT-PCR performed with an exogenous internal control was obtaining a false-negative result. One time a sample's result did not show any amplification in the virus's genes and showed an amplification in the internal control. This led us to believe that the sample was negative. However, when we amplified the same extracted RNA using an amplification kit with an endogenous internal control, the internal control was not amplified. We concluded that the sample did not contain enough cells and the swab collection was probably not properly collected. We ordered a new swab for the patient, which turned out to give a positive result.

We note here that sometimes when the sample is positive, the internal control is not amplified. This may be as the amplification of the virus's genes interferes with the amplification of the internal control's genes.

We also emphasize checking the cycle threshold (C_T) value of the internal control, which is very important during analysis. Cycle threshold is defined as the calculated cycle number at which the PCR product crosses a threshold of detection. A C_T value for the internal control greater than 35, for example - this differs from one kit to another – means that a lot of cycles were required in order to detect any amplification. This indicates insufficient or improper extraction and that the sample's extraction should be repeated. We recommend following the kit's instructions regarding which C_T values are acceptable for analysis.

Although we were analyzing a patient's result that appeared to be negative, we noticed that the C_T of the internal control was 36 while using Bosphore amplification kit. After repeating the extraction and amplification of this sample, the result turned out to be positive whereas the new internal control of the C_T was 30.

It is also worth mentioning that a Nano-drop could help in measuring the quality and quantity of the extracted RNA.

Analysis of the curves

We also focus on the shape of the obtained curves during result analysis. For the results to be considered reliable, the shape of the curves has to be sinusoidal. There were times when some wells showed amplification curves in the virus's genes having C_T values around 36, 37 (Fig. 1). However, the shape of the curves was almost straight rather than sinusoidal so we suspected they were unspecific curves. We repeated the amplification of these samples from the same extracted RNA but using a different amplification mixture. The results turned out to be negative and the previous curves disappeared. We have noticed that this happened after thawing–freezing the amplification mix several times over a long period of time. This has also happened with certain batches of amplification kits that may have had sensitive or unstable reagents, disproportional oligonucleotide design, or possible impurities.

At other times, such unspecific curves might be observed throughout the entire plate, including the negative control, even after the very first use of the master mix (Fig. 2). In such cases, we suspect contamination and repeat the extraction of the samples corresponding to the wells showing such curves. In parallel, we repeat the amplification of the extracted RNA using a different amplification kit. If the same extracted RNA did not show any amplified curves using a different mix,

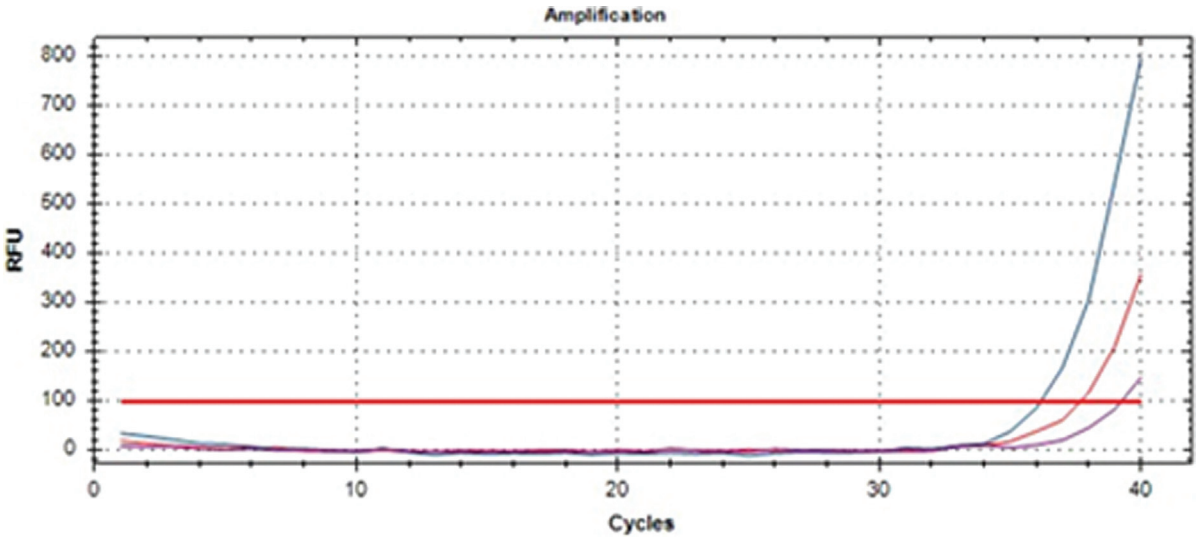

FIGURE 1. Amplification plot showing an unspecific curve.

then we can rule out contamination. We theorize in this case that these curves may be because of the spontaneous dis-integration of the quencher from the fluorophores, thus releasing a fluorescent signal even if genes were not present.

To ensure the integrity of the master mix, we suggest dedicating a well in the amplification plate for the master mix alone or for the master mix loaded with nuclease free water. This well is not supposed to give off any fluorescence or curves as it lacks a sample and was not involved in the extraction process.

It is also important to remember that the primer/probe sensitivity differs from one kit to another. The same positive sample may display a result of $C_T = 23$ (Fig. 3a) using one kit and $C_T = 18$ (Fig. 3b) using a different kit. This has posed a problem as some patients perform the PCR test in the same day at different labs. A positive patient with a low viral load (e.g. $C_T = 33$) who

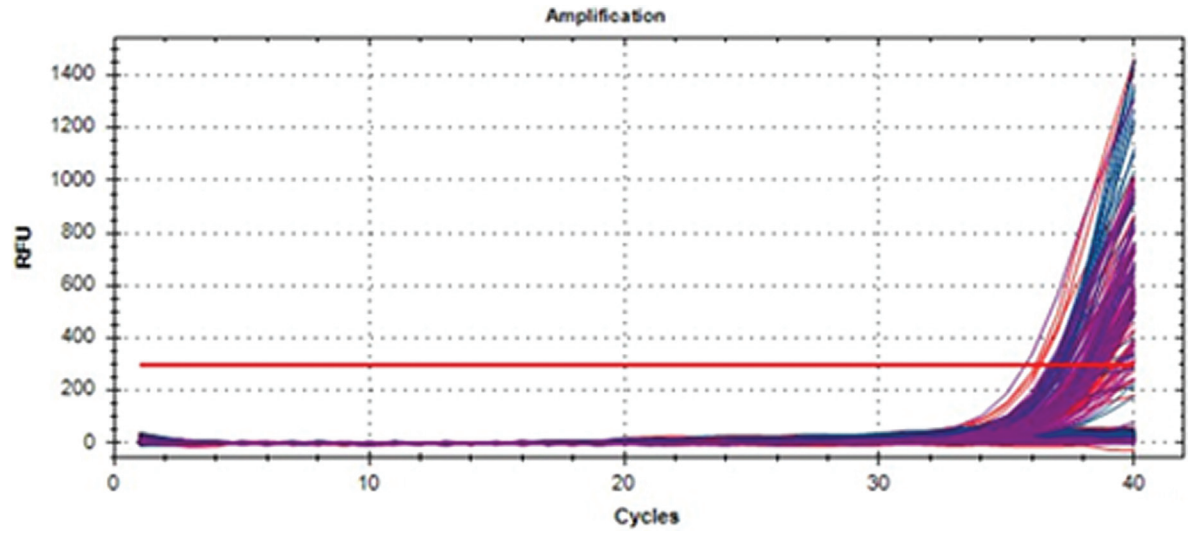

FIGURE 2. Amplification plot showing bulk of unspecific curves for several samples.

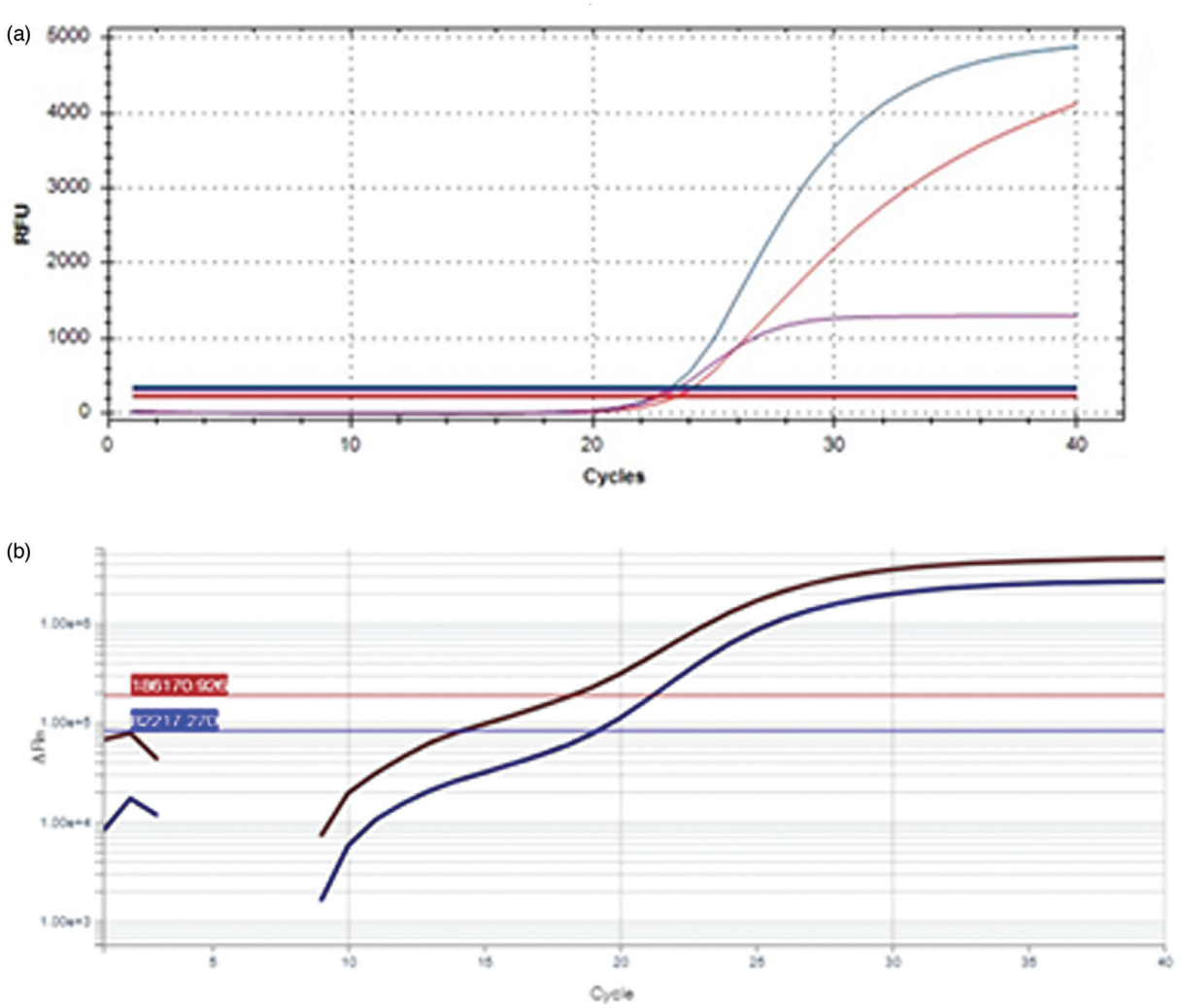

FIGURE 3. Amplification of a positive sample using two different kits. (a) A positive sample of C_T 23 using Bosphore kit. (b) A positive sample of C_T 18 using TaqPath kit.

repeats the test at a different lab that uses a different kit may test negative.

Conclusion

The RT-PCR technique has become an indispensable tool in COVID-19 detection. Accurate detection and quarantine of positive cases plays a huge role in containing the pandemic and limiting the morbidity and mortality rates that result from it. Therefore, both the procedure and result interpretation must be very carefully done. Thus, following the correct swabbing and lab protocols, using adequate RT-PCR kits with suitable controls, and thorough result interpretation all reduce unreliable results and help with the appropriate containment and management of this disease.

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Conflicts of interest

There are no conflicts of interest.

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