Does the absence of SARS-CoV-2 specific genes always exclude the infection? How to interpret RT-PCR results?—The scenario of interactive online workshop

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Abstract

The aim of this online workshop is to familiarize biomedical faculties students with the principle of RT-PCR method. The following assumption is made, students participating in the workshop:

1. are already familiar with the principle of PCR reaction,
2. can distinguish PCR from RT-PCR,
3. know the basic possibilities of using the above techniques.

During the online workshop participants are supposed to learn the interpretation of PCR and RT-PCR results and to understand the crucial importance of controlling the reaction conditions. The workshop involves active students’ learning, critical analysis of the data, group discussion, brainstorming method, involvement of e-tools such as pool everywhere or e-learning platforms, as well as interpreting the real-life example results that allows putting the topic in the proper future work-related tasks. The final part of the workshop focuses on the analysis of the RT-PCR results performed in order to confirm or exclude the presence of the SARS-CoV-2 genome in potentially infected individuals. The students are expected to see the practical/work-related part of the knowledge gained during the workshop.

KEYWORDS

RT-qPCR, RT-PCR controls, SARS-CoV-2

1 | INTRODUCTION

The following article presents the scenario of the online workshop for biomedical and medical faculties’ students, which can be applied as an introduction to practical biochemistry lab classes following the theoretical course in basic biochemistry. Performed in the designed protocol provides not only the better understanding of the PCR/RT-PCR reaction principle, but also shows how to interpret the obtained results. During the theoretical biochemistry courses students gain the basic knowledge regarding reverse transcription, stages of the polymerase chain reaction, distinction between PCR, qPCR, RT-qPCR as well as general usage of PCR in medical diagnostics. However, the acquisition of the theoretical knowledge does not always allow to use it in the practical aspects, nor does it give the ability to properly interpret the obtained results. PCR-based research is the center of molecular diagnostics in medicine. Thus, it seems to be justified to introduce the active workshop model of learning it for students. In our learning model, the proposed online workshop should be the one preceding the laboratory classes, where the manual part does not always allow for sufficient discussion of more complex and
difficult theoretical aspects. After the workshop students should gain the abilities required to compile the Intended Learning Outcomes (ILO) (Table S1).1,2

1.1 | Part 1

1.1.1 | Part 1A

During the vacations Emma, Christopher, and Alex worked in the laboratory. Their first task was to analyze the MGMT gene expression in biological material (obtained from oral cancer biopsy) of three patients (P1, P2, and P3). MGMT gene expression is often lost in cancerogenesis what may influence the response to alkylating agents used in chemotherapy. After the correct application of the samples on the gel and electrophoresis, the students did not obtain any bars (RT-PCR products) except for the separated marker (M) (Figure 1). What can be the interpretation of the result obtained by the students? At the beginning of the on line workshop all students receive the result of MGMT gene expression analysis. The material represents a real-life based example, and all three samples represent the result for the patients. The photograph of the gel separation shows a separated marker, but no product for the MGMT gene expression. There is also a result for the negative control (interpretation of the result, no presence of the band in the negative control indicates no contamination during the RT-PCR procedure). In this section of the workshop—students try to find the explanation for the lack of an RT-PCR reaction product within the presented patients’ results. Students can put forward several reasons, as at this stage of the analysis the simplest conclusion about the lack of expression is impossible to confirm unequivocally. The proposed form of this part of the workshop will be a brainstorming. Each student tries to find as many interpretations as possible to contribute in the final conclusion. In addition to facilitate this part we propose the interactive online pool of answers: poll-everywhere, that will facilitate the proposed method in virtual character of the workshop (introducing a modification of brainwriting to the general idea). Thanks to this tool students can propose their ideas remotely and the whole pool of answers can be seen on teachers’ screen and commented in the real-time manner. Each student will be asked to create their own checklist on the base of the created pool. An additional advantage is the possibility to up-vote the seen results during the discussion. Each student in the time when the pool is active can vote for the best answer. The teacher additionally may pose the question such as: “Can we say that since we do not observe the bands at the appropriate height in the P1, P2, and P3 samples tested, there is no MGMT gene expression? Altogether it will introduce also a second modification of the proposed method inverted—brainstorming and allow for more interactive character of the remote teaching/teacher student online interaction. Along with the ongoing discussion it will help to eliminate the most general and commonly given reason for this problem—the lack of expression—and acknowledge the two general problems regarding the mistakes in the methodology at the level of RT stage or PCR stage as well as lack of the controls. In general, regardless of the number of potentially possible causes, the interpretation of the results may be divided into two categories: RT stage error (Table 1 left column) or PCR stage error (Table 1 right column). After gathering all the student ideas, it is worth showing the students that all their proposed interpretations of the result conclude to these two categories (Table 1).

1.1.2 | Part 1B

In the next PCR reaction, the students additionally performed the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression analysis at the same time as the MGMT gene. What can be the interpretation of the result obtained by the students? Which options (from the previous conclusions) can be now excluded and why? The following part presents the result with a positive GAPDH (housekeeping gene; Figure 2) gene expression obtained. Students could start the analyses from the table/checklist.
They should note that all interpretations regarding the cDNA preparation errors can be excluded (Table 1, left column). The interpretation of the results from this section allows to observe/to conclude that housekeeping gene control represents also a quality control of the obtained cDNA. This part of the workshop also addresses the selection of the appropriate housekeeping gene as well as the fact that external factors in the experimental system should not affect the expression level of the selected gene(s).

Table 1: The possible explanation for the lack of PCR bands appearance in Figure 1

| Reverse transcription protocol (RT) mistakes (eliminated by application of housekeeping gene (GAPDG) analysis (Figure 2)) | PCR protocol mistakes (eliminated by application of PCR positive control (Figure 3)) |
|---|---|
| • Faulty RNA sample preparation | • DNA polymerase was stored incorrectly and lost its activity |
| • RNA was already degraded (before the reverse transcription stage | • In the PCR reaction there were two identical starters used instead of the forward and reverse pair |
| • Degraded RNA due to the presence of RNase | • A program not suitable for MGMT was used in the thermocycler |
| • The reverse transcriptase was not kept on ice and the enzyme had lost its activity | • Temperature, number of cycles or/and time choose for the thermal cycler programming was set incorrectly |
| • There were no oligo dT added during the reverse transcription reaction | • The reaction mixture was incomplete |
| • There was no RNA present for cDNA preparation | • Primers were designed incorrectly |
| • RNA was contaminated with compounds that inhibited the reverse transcriptase | • No primers were added |
| • The work was conducted without a proper protection such as gloves (allowing the RNA degradation by e.g. RNAses) | • The amount of Mg^{2+} ions was not optimal |
| • Temperature and time set for the thermal cycler was set incorrectly | • Annealing temperature was badly chosen |
| • The RT mixture was incomplete | • DNA polymerase was stored incorrectly and lost its activity |
| • RNA was not added to the mixture | • In the PCR reaction there were two identical starters used instead of the forward and reverse pair |
| • RNA quality was not checked (it could undergo degradation) | • A program not suitable for MGMT was used in the thermocycler |

The third PCR reaction performed by students, included additionally the analyses of the expression of the MGMT and GAPDH gene in a cDNA sample from the previously checked high expression MGMT cell line. What can be the interpretation of the result obtained by the students for this last experiment? Which options (from the previous conclusions) could be excluded and why? The next part of the workshop is focused on the complete result analysis. The students are presented with the picture of RT-PCR result with the presence of positive control, negative control, marker and patients results (Figure 3). The band for a high expression MGMT line (positive control) allows for the conclusion that the PCR procedure was correct, and all the reasons presented in Table 1 (right column) can be eliminated. Students see that only with both: cDNA quality control (house-keeping gene expression) and positive control the conclusion can be drawn. All entries presented in Figure 3 lead to the conclusion that there is no gene expression in the P1, P2, and P3 samples due to the lack of RT-PCR product on gel. At this point of class, it is also worth further, detailed discussing that the validity of the negative control is crucial for result interpretation (confirms the lack of contamination of the reagents used at each stage of the procedure). Students should also be aware that obtaining a positive result/a band in the negative control does not allow for interpretation of the analysis results and clearly indicates a mistake at some point of the procedure. After the conclusion of/summing up Part One, students will be
involved in the part of the workshop allowing them to see how to acquire the knowledge that can be used in everyday life. All the mentioned adequate positive and negative controls are just as necessary to interpret RT-qPCR technique results, routinely used to test for viruses with RNA genome as SARS-CoV-2. The classic RT-PCR model with the detection of the PCR products in agarose gel (as it was described and discussed in the Part 1A of the workshop) is a very simple and clear model for analysis and allows to reach the didactic goal of understanding the validity of the applied controls. The workshop is designed in such a way what it does not involve a detailed investigation in the RT-qPCR method/procedure/analysis. In order to interpret the results of SARS-CoV-2 virus genome the knowledge gained in the first part of the workshop is sufficient. However, the second part can be used to further discuss the principles of multiplex RT-qPCR used is SARS-CoV-2 diagnostics.

1.2 | Part 2

The students begin the second part by discussing the necessary controls to analyze the presence of the SARS-CoV-2 genome in a potentially infected patient. At this stage they should already know that there is a need for a positive control for each virus gene under investigation to confirm a proper PCR procedure, a negative control to confirm the purity of the reagents/laboratory work, and a control of RNA isolation and reverse transcription steps of the experiments. For SARS-CoV-2 virus analysis, an internal control for isolation and reverse transcription is used. It is RNA for RNase P that is added externally at the laboratory procedure stage. At this stage of the workshop the students may also consider the possibility of viral RNA (potentially present in the sample) degradation by the RNase P—a ribozyme. According to the literature data, such a degradation is not possible. The RNase P exhibits no catalytic properties without a protein presence when not combined with the protein. After discussing the necessity of the controls usage students are provided with a data/information collected in Table 2. Their task is to interpret it and try to determine whether the final result will be classified as positive, negative or inconclusive. The proposed form of this part of the workshop will be a small-group work with the usage of/in an online application that allows to divide students into small groups (online platforms like Zoom allow for creation of “Breakout rooms” where teacher may split your Zoom meeting in up to 50 separate sessions. Teacher may split the students into these separate sessions automatically or manually). Each group will discuss the results in the given amount of time, construct the interpretation in the written, graphic or oral form and conclude with the presentation of the interpretation by the group leder. Students are given a combination of nine possible results for analysis. The assumption about the validity of the positive control for all tested genes (PCR positive control for viral genes and RNase P) and the negative control (excluding contamination at the procedure stage or reagent preparation—PCR negative control for viral genes and RNAs P) is included in the instructions. In this particular case the negative control may constitute a blank sample where, instead of a matrix, a RNase and DNAfree water and/or a negative test sample/probe containing RNA for RNase P is present (acting as a control of extraction and reverse transcription). Thus, allows for the control of the whole process at each step of the laboratory protocol. Three genes, one Sarbecovirus (E gene) family

![Image](image-url)
specific and two SARS-CoV-2 viruses specific (RdRP or N gene) were analyzed together in an RT-PCR reaction using different markers (Multiplex PCR). At the same time, positive control was applied for isolation and reverse transcription (RNA for RNAse P). The description of results possible to obtain in COVID-19 multiplex RT-PCR is given below and additionally the summary of results is presented in Table 3. The requirement for obtaining a confirmed positive result in the COVID-19 multiplex PCR technique is the detection of at least one SARS-CoV-2 virus-specific gene.6,7

- The first report (No. 1)—the SARS-CoV-2 virus is present—the positive RT-PCR result for three virus genes and positive or negative result for RNAse P—indicates unequivocally the presence of the virus genome in the examined material. For all three detected genes in the sample, the potential lack of the positive results for RNAseP points to a problem with an external control addition that is not related to the taken smear sample.
- The results from 2 to 6 (Nos. 2–6)—the SARS-CoV-2 virus is present—the positive RT-PCR result for/two genes were detected in samples 2–4. Whether the two
genes are SARS-CoV-2 RNA specific (N-gene and RdRP-gene) or only one gene is specific (N-gene or RdRP-gene) when the other confirms the presence of Sarbecovirus RNA (E gene), the result is considered as a positive;

- In samples 5 and 6, the detection of only one of SARS-CoV-2 specific genes (RdRP or N gene), despite the absence of the other, is considered as a valid result. Various factors may affect the lack of gene expression, such as insufficient RNA concentration for the detection of a specific gene.
- The result 7 (No. 7)—the negative/no SARS-CoV-2 virus present - RNA for SARS-CoV-2 (COVID-19) was not detected. The lack of expression of the three tested genes with simultaneous detection of the RNA for positive control indicates the correct RT-PCR reaction procedure.
- The result 8 (No. 8)—the doubtful result/ the result need to be repeated—a negative result for two SARS-CoV-2 specific genes (N-gene and RdRP-gene), even in the presence of the third Sarbecovirus specific RNA gene (E gene) indicates that the result cannot be interpreted properly. The result confirms the presence of Sarbecovirus RNA only. The sample did not meet the full criteria established for SARS CoV-2 RNA detection (COVID-19) within the used test. The result should be repeated using the same or an alternative method.
- The result 9 (No. 9)—the negative result/the result need to be repeated—a negative result for all three genes could confirm that SARS-CoV-2 RNA was not detected, but the absence of RT-PCR reaction control for RNAse P excludes further/proper analysis of this result. In this case, it is not possible to determine whether the patient’s result is positive or negative for the presence of SARS-CoV-2 because the isolation and/or reverse transcription reaction has not been performed correctly.

2 | CONCLUSIONS

Principally the workshop is intended to show students that the analysis of the result itself can only be interpreted in the light of correct method controls. Secondly it focuses on the analysis of the real-life and future work-related examples which enables the student to see the importance and context of the gained knowledge. During the classes, students also learn to discuss the results, how to think critically, reason and work in the group. The topic of SARS-CoV-2 virus detection seems to be an excellent and universal model for building a lesson scenario and can be used regardless of the current state of the pandemic. The workshop, in line with the described scenario, has been already conducted by the authors during online classes with students of the 2nd year medical and 3rd year dentistry faculties. It was enthusiastically received by the students and put the RT-PCR method topic in a real-life current context. Undoubtedly, the pandemic situation influenced the level of students' interest in the workshops, but this model may be successfully used for several years, as the problem of the pandemic will definitely remain valid for quite a long time.

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