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Letter to the Editor

Troubleshootings in RNA extraction – back to basic chemistry

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Dear Editor,

Real-time reverse transcription polymerase chain reaction (RT-qPCR) is a PCR-based technique used to detect and to quantify RNA in real time. With the increasing availability of real-time PCR systems, RT-qPCR has become the tool of choice for RNA detection and is nowadays available for routine diagnostics. Since December 2019, RT-qPCR has seen a dramatic increase in use worldwide as it has been and continues to be the main diagnostic method for the pandemic novel coronavirus disease 2019 (COVID-19) which is caused by the RNA virus SARS-CoV-2 (1).

As RNA is considerably more vulnerable to enzymatic degradation than DNA, RT-PCR requires additional caution to preserve the target RNA throughout the extraction process (2). When amplification failure for the internal control (IC) occurs, the test result is nonvalid and the source of error must be identified. RT-PCR failures can be caused by problems in both the extraction and amplification phases and the sources of error are manifold: biological sample particularities, inappropriate collection container, human error, contamination with ribonuclease (RNase) or various PCR inhibitors, extraction/amplification kit problems, inappropriate or damaged reaction plates, malfunction of the RT-PCR machine, etc (3). However, the reason for RT-PCR failure is usually rapidly identified. Here we describe our RNA extraction and RT-PCR troubleshooting algorithm along with an unexpected finding.

Our department is currently working exclusively in COVID-19 diagnosis using RT-qPCR. After a brief period of testing without any incident, 3 nonvalid test results occurred in one of the daily runs, followed the next day by another 7. During the following two weeks, a variable number of amplification failures kept occurring, affecting each member of the extraction team at least once. Given that most of the extraction team recorded only random and scarce amplification failures, occasional and isolated nonvalid results were not considered a problem in practice, neither proof of systematic error. However, some of our less experienced users displayed a consistent pattern of PCR failure occurrence, suggesting that the problem was, at least partially, user dependent. A crushing argument was that re-extraction from the same specimen by a more experienced user, in the same extraction/amplification conditions was always successful, resulting in proper amplification. Despite our troubleshooting efforts described in Table 1, amplification failure kept occurring systematically for certain users without apparent cause.

Two weeks later, a staff member working in the amplification area noticed a variation in the volume of the eluate between samples: some of the tubes contained a visibly larger eluate volume than the others. After the amplification was over, we were surprised to see that the nonvalid test results in that run were corresponding to those exact samples where a larger eluate volume had been previously noticed. Also, RNA extraction for all these samples had been performed by one of the less experienced users. Naturally, we concluded that incomplete elimination of the solutions used in the pre-elution steps was most probably responsible for the observed excess eluate volume. Since elution was preceded by a washing step with pure ethanol, which was also the main component of the washing buffer pro-

Table 1. Troubleshooting algorithm to identify the source of amplification failure

Suspected problem	Troubleshooting	
1. Pre-analytical errors	Rationale	Specimen particularities, collection technique, container type, time, storage and transport can adversely affect laboratory test results
	Cons	a) Pre-analytical errors are not (extraction) user-dependent b) Re-extraction from the same specimen by a more experienced user always worked
	Measures	Collection of a new specimen
2. Contaminated, defective or inappropriate extraction equipment	Rationale	RNase is ubiquitous. Heavy RNase contamination of the extraction area equipment may result in IC/sample RNA degradation. Defective/inappropriate equipment may facilitate the contamination.
	Cons	a) The same equipment is used by all users and, if defective/contaminated, should affect all users equally b) The equipment is properly maintained
	Measures	a) Additional equipment decontamination b) Equipment revision c) Cross-testing the equipment between users
3. PCR inhibitors	Rationale	There are various PCR inhibitors whose presence results in amplification failure for both IC/sample even in the presence of IC/target RNA material
	Cons	Amplification failure caused by PCR inhibitors is not user-dependent unless the user is the source (unlikely).
	Measures	Checking laboratory equipment/environment and extraction/amplification kits for known PCR inhibitors
4. Extraction kit, amplification kit, reaction plates, PCR machine	Rationale	Inappropriate or damaged reaction plates or extraction/amplification kits, as well as defective PCR machines may result in amplification failure
	Cons	a) The extraction/amplification kits and reaction plates were apparently intact and properly stored b) Amplification failure caused by such problems is not user-dependent c) Most of the tests performed using the same kits/plates/machine were valid
	Measures	a) Extraction/amplification kits and reaction plates re-examination b) Storage conditions re-evaluation
5. Human error	Rationale	PCR is a sensitive technique with much room for human error at each step. RT-PCR is particularly challenging due to the vulnerability of RNA during the extraction phase. Inappropriate RNA extraction technique may result in undesired contamination and amplification failure.
	Pros	a) Amplification failure caused by human error during the extraction phase is user-dependent b) Amplification failure systematically occurs only for certain users
	Measures	Only for the users who systematically recorded PCR failures a) further training for RNA extraction b) workspace optimization c) contamination assessment using dyed samples d) working under supervision

vided in the extraction kit, it was safe to assume that the eluates at issue were contaminated with a considerable amount of ethanol. As our laboratory had no routine or readily available method for identifying ethanol in aqueous solutions, we had to resort to the old chemistry cabinet. The eluates in question were tested by means of an old school color change redox reaction. An aqueous solution of potassium dichromate ($K_2 Cr_2 O_7$, orange) was prepared and added to the remaining eluates from the previous run. Upon addition of a strong acid to the mixture, at room temperature, the eluates which had larger volumes turned green in less than 3 minutes and a specific odor of acetic acid emerged (4). On the contrary, none of the samples that had had normal eluate volumes were positive to ethanol testing. Further tests were performed the following days, all pointing to the same conclusion: some of the eluates from our less experienced users had volumes up to 2× larger due to ethanol contamination and these samples always produced nonvalid test results. Ethanol, especially in high concentrations, is a well-known PCR inhibitor (5) and its presence in the eluate explains the amplification failure. However, given a proper and standardized RNA extraction technique, the reason behind systematic ethanol contamination continued to puzzle us.

While still troubleshooting, we had to prevent ethanol from contaminating the eluate. Increasing the centrifugation time and/or centrifugal force at key points during the protocol may have helped to remove residual ethanol from the spin column, if any. Nevertheless, the temporary solution that completely solved the ethanol contamination problem for us was to perform a second elution step and use the final eluate for amplification as silica-based spin columns are known to retain residual nucleic acids after the first elution (6). However, Cq delay in PCR is bound to occur and the Cq thresholds must be adjusted accordingly. In our experience, amplifying the second eluate has solved the problem at the cost of a 2Cq delay (constant). In theory, additional Cq delays could lead to false-negative test results in samples with low viral RNA levels. However, in practice this is never the case since virtually all commercial RT-qPCR kits for SARS-CoV-2 detection require that the number of amplification cycles be well above the positivity cycle threshold (e.g. a cycle threshold value less than 35 is defined as a positive test result, but 45 amplification cycles are required).

Interestingly enough, after another couple of weeks, contaminated eluates were observed only occasionally, thus suggesting that user experience was an important aspect. Despite this, we continued our quest for the source of ethanol contamination. Nucleic acid extraction is mainly based on following the extraction protocol, but each user has its own unique approach. The individual extraction technique can be regarded as the sum of all perceptible/imperceptible and conscious/unconscious motions and gestures performed by the user during extraction. Consequently, a faulty gesture that is unconscious and imperceptible may result in sample contamination that can elude both the user and their supervisor. Therefore, having repeatedly excluded all major and obvious errors, we focused in turn on the slight differences in extraction technique between users. After further investigation, we presumed that ethanol contamination was caused by improper handling and removal of the spin column (Sc) and receiver tube (Rt) from the microcentrifuge just before the elution step. Given the close gap between the bottom of the Sc and the ethanol/wash solution collected in the Rt, we considered that any sudden acceleration/deceleration or excessive tilting of the Sc-Rt may, in theory, result in pre-elution contamination of the Sc. In order to test this theory, a series of mini-experiments was performed using Sc of different designs from 4 distinct providers.

Improper handling of the Sc and Rt was simu-

lated by 1) dropping the Rt back into its microcentrifuge slot after an almost complete removal (simulating a bad grip/slip) and 2) accelerating/decelerating and overtitling the Sc-Rt after removal from the microcentrifuge (simulating a hasty behavior/unrefined technique). We found that small traces of ethanol can be found even in properly handled samples, but this should not cause PCR inhibition. Improper handling of the Sc-Rt before elution may result in backflow contamination of the Sc membrane and outer walls. Upon elution, the contaminating liquid will end up in the eluate. If the contamination is significant, the eluate volume is visibly larger, but a visibly normal eluate does not exclude the possibility of PCR failure due to contamination. Apparently, the contamination of the Sc outer wall is more significant and will probably result in higher eluate volumes and PCR failure. However, membrane contamination only may be enough to inhibit the PCR. Such backflow contamination is probably the reason why some commercially available extraction kits recommend an additional/optional centrifugation step using a new receiver tube just before elution.

In conclusion, if PCR failure occurs systematically, a general troubleshooting, similar to that presented in Table 1, must be done. If the problem is user-dependent, increased awareness of own extraction technique at each step is advised. Beginners should not be discouraged by repeated failures. Individual practice in a stress-free non-diagnostic environment may help speed up the troubleshooting process. A key moment for preventing contamination is the pre-elution step. If backflow contamination is suspected, an additional "dry" centrifugation using a new receiver tube should remove any contaminating liquid from the silica membrane/spin column walls.

Finally, we would like to summarize our experience in the form of 5 key points:

- Amplification failure sometimes occurs without apparent cause and despite proper training; individual experience and practice are crucial
- When the source of error is user dependent, troubleshooting can become a lengthy and complicated process
- User-dependent subtle variations in extraction technique may result in contamination of the eluate with PCR-inhibiting substances like ethanol
- If such contamination is suspected, visual examination of the spin column before elution may reveal backflow contamination liquid; visual examination of the eluates may reveal abnormal volume variations
- Manually performed basic chemical recognition reactions can still be useful in the era of automation and should not be forgotten.

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Abbreviations

COVID-19 – novel coronavirus disease 2019
Cq – cycle quantification
DNA – deoxyribonucleic acid
IC – internal control
PCR – polymerase chain reaction

q – real-time
RNA – ribonucleic acid
RNase – ribonuclease
RT – reverse transcription
Rt – receiver tube
Sc – spin column

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Authors' contributions

IBM (Conceptualization; Investigation; Methodology; Validation; Visualization; Writing – original draft preparation)
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Conflict of Interest

The authors declare that there is no conflict of interest.

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