



Short communication

Pitfalls in PCR troubleshooting: Expect the unexpected?

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ABSTRACT

PCR is a well-understood and established laboratory technique often used in molecular diagnostics. Huge experience has been accumulated over the last years regarding the design of PCR assays and their set-up, including in-depth troubleshooting to obtain the optimal PCR assay for each purpose. Here we report a PCR troubleshooting that came up with a surprising result never observed before. With this report we hope to sensitize the reader to this peculiar problem and to save troubleshooting efforts in similar situations, especially in time-critical and ambitious diagnostic settings.

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1. Introduction

During the past years PCR has become the gold standard for the molecular diagnostics of manifold diseases, such as cancer or infectious diseases. In most cases real-time PCR applications have replaced conventional PCR tests due to their obvious benefits, namely speed, increased specificity, (semi-) quantification, reduced work-load, and a minimized risk of carry-over contamination, which is of benefit especially in diagnostic labs [1,2].

Consequently several standardized PCR kits for most clinically important and frequently needed molecular targets have been made commercially available by several manufacturers. However, there are at least as many PCR applications in the field of PCR diagnostics for which there is no real market and unfortunately no commercially available kits are being developed. This applies for example to the field of neglected as well as new and emerging infectious diseases. For these issues usually in-house assays have to be established and validated by expert laboratories, which can only be as good as it is technically feasible regarding the often low isolate numbers available.

The manufacturer and the specifications of PCR components used in in-house PCR assays have to be selected by the user who, in general, relies on his previous experience. Components that are common to any PCR and are present in the basic reaction mix are the enzyme for PCR or RT-PCR, the buffer required for the enzymatic reaction, Mg²⁺ or other bivalent ions, dNTPs or derivatives, water, and finally as target-specific ingredients primers and probes.

The basic reaction mix can be set up from all mentioned individual components. In this context, there is a kind of unwritten law that everyone who has set up PCR reactions by themselves has experienced trouble with amplifications that somehow unexplainably fail. The reason for PCR failure is usually rapidly identified. Often problems can be explained by the fact that essential components like Mg²⁺ ions or even primers were of poor quality because the expiration date has passed or were unintentionally not added to the reaction mix. The PCR set-up is then started from scratch with new aliquots of components and in most cases the problem is solved. A well-accepted approach to reduce the risk of making an error in preparing the basic PCR mix is the utilization of a ready-to-use mastermix that contains all components except for the primers and the probe(s). These ready-to-use mixes have become popular since they contribute to a certain level of standardization and speed up and ease hands-on times.

However, also ready-to-use basic mixes can be subject to a varying PCR performance and have to be controlled for functionality. For example, in compliance with quality management, a new batch of a reagent has to be compared with the old one in the same run with the same assay. As long as the results obtained are not different, the new reagent batch is accepted as fully functional.

Here we describe a PCR “case report” with unexpected results in a routine PCR troubleshooting.

Our division deals with the diagnostics of various highly pathogenic viruses, with real-time PCR as the most popular approach. One pillar of this diagnostic portfolio is one-step real-

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Table 1
Troubleshooting of the failed PCR reaction for Lassa virus [5].

Suspected reason	Measures	Amplification
Human error	Repetition of all PCRs	No
Failure of the positive control	Use of new aliquots of <i>in vitro</i> transcribed RNA and plasmid DNA also highly concentrated	No
Primer and probe damage	Use of fresh primer and probe aliquots resolved and previously tested to be working properly	No
Primer and probe damage	Use of fresh lyophilized aliquots of primers and probes	No
Problem with the basic reaction mix from manufacturer A	Use of new aliquot of the basic one-step RT-PCR mix from manufacturer A (although the aliquot used proved to work for YFV RT-PCR)	No
Inhibiting influence of water	Test of freshly opened PCR water aliquots	No
Individual problem of the cyclor	A different ABI 7500 cyclor was used (although the used one proved to work for YFV RT-PCR)	No
Problem of the ABI7500 series	Parallel PCR runs on various real-time PCR cyclors including ABI 7500, ABI StepOne™ and Roche LightCycler® 480	No
Individual problem with the Lassa virus assay	Application of another LASV-specific RT-PCR targeting a different region of the same gene	No
Problem with the PCR enhancer	Parallel PCRs with and without enhancer	No
Problem with the reaction plates	Use of plates from a different manufacturer	No
Problem with the basic reaction mix from manufacturer A	Use of a basic one-step RT-PCR from another manufacturer B	Yes
Problem with a specific batch of the basic reaction mix from manufacturer A	Use of remaining failing, old aliquot of the preferred basic one-step RT-PCR mix from manufacturer A	Yes

time RT-PCR-based detection of viruses causing hemorrhagic fever, like Ebola virus (EBOV), Marburg virus (MARV), Lassa virus (LASV), Yellow fever virus (YFV), and viruses relevant for differential diagnosis. For those viruses, for which there are no commercially available kits and also for confirmation of positive results obtained by using kits, we apply in-house tests using a basic one-step RT-PCR reaction mix from a manufacturer A. These assays are thoroughly validated with this reaction mix. According to quality management rules, we always compare a new batch of the basic reaction mix with the old one. Since there are currently about 40 validated in-house RT-PCR assays in our portfolio, not every single assay is subjected to comparison, but some in-house assays are selected exemplarily and compared.

2. The case

In a routine request to exclude relevant viral hemorrhagic fevers in a hospitalized patient we received serum to run PCRs for EBOV, LASV and YFV. Usually these requests are urgent, due to the public health precautions potentially required for highly contagious patients. RNA was extracted from serum using the Viral RNA Mini Kit to which the Internal Control (IC) provided with the RealStar® Filovirus Screen RT-PCR Kit 1.0 (Altona Diagnostics) was added according to the manufacturer's instructions. As negative control, Fetal Calf Serum (FCS) was extracted and spiked with the IC in parallel. We performed the EBOV PCR using the Filovirus Screen Kit, obtaining a negative result within 2 h. The IC showed perfect amplification curves with similar C_q values of about 31 for the clinical sample as well as for the FCS control, thus confirming successful extraction and RT-PCR reaction and also ruling out sample-specific PCR inhibition. In parallel, we set up in house one-step RT-PCRs for the detection of YFV and LASV using published assays [4,5]. For these assays we routinely use a basic one-step RT-PCR reaction mix from manufacturer A. Both assays were set up in parallel using the same aliquot of water and reaction mix and were run in parallel on the same Applied Biosystems 7500 Real-Time PCR Instruments. The sample was negative for YFV with perfectly fine amplification curves for the positive control (*in vitro* transcribed RNA). In contrast, the LASV assay did not show any amplification, neither for the sample nor for the positive control. Even on an agarose gel no amplification of the positive control could be shown. Since these results could not be explained easily, we started troubleshooting

by checking 12 parameters [3], which were sometimes combined in one run. Table 1 summarizes the troubleshooting activities.

As shown in Table 1, all obvious possible measures were taken without success. Even measures that were not the first choice failed to restore the PCR assay. Only when we used a basic one-step RT-PCR reaction kit from another manufacturer B, we could observe amplification as expected. At this point we need to point out that the Lassa virus PCR assay was well validated with the preferred basic PCR kit from manufacturer A which turned out to be superior to the kit from manufacturer B in terms of detection limit in our validation process. Hence, this result was unexpected and surprising. However, it led us to the idea to test an aliquot from an old batch of the kit from manufacturer A and we were again surprised that this old aliquot worked as expected.

While the old batch worked for every single one of our assays tested, the new batch only worked for some assays, e.g., the YFV assay [4], but not for others, e.g., the LASV and West Nile virus assays [5,6].

Interestingly, considering the rules for PCR design and assessment, we were unable to identify any physical parameter of the primers like T_m, GC-content, Hairpin T_m or degree of degeneracy that would allow us to predict the sensibility of an assay to obviously slightly changing reaction conditions with the new batch.

As described above, comparing old and new batches always controls a change of a reagent's batch. However, not all of the 40 assays can be compared, for economic reasons and because of a restriction in batch size and stability. So far, we have been assuming that the proof of functionality of a new reagent batch for an assay selection is sufficient evidence of the quality of the new reagents batch. This does not seem to be true for all individual assays. Therefore we wanted to share the lessons learnt with the PCR community, particularly with those who need to rely on rapid PCR-based diagnostic results, hoping to add a new troubleshooting criterion to the recent check list.

According to the manufacturer of kit A, the new batch passed the QM check and no other customers reported similar problems. However, fair enough, the company fully compensated the lost reagents.

3. Conclusions

Summing up, we found that (i) different PCR assays show individual sensitivity toward changes in the reaction mixture. Again, we

are not talking about new reaction conditions, but about expectedly the same reaction conditions and just a batch change of the same product. (ii) Since some of the assays were absolutely identical in performance after the batch change, conclusions drawn on one assay can not be transferred to another one. (iii) The detrimental effect of a new batch can result in a complete failure of amplification. Minute changes in buffer substitutions usually result in reduced PCR performance, but not in a complete failure. (iv) Batch changes should be controlled individually for every assay. To reduce costs and workload, large batches of the respective reagents should be purchased. (v) Preparing important PCR assay protocols for reagents from more than one manufacturer may help to assure rapid reliable diagnostics. In our case, we were still able to report the negative result in time.

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