Review

Standardization and Quality Control of PCR Analyses

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In the very beginning of polymerase chain reaction (PCR) tests entering the field of diagnosis of infectious agents, the introduction of this technology into routine diagnosis was hampered by its frequent tendency to create false-positive results because of contamination. This problem is now widely solved by the introduction of the uracil-N-glycosylase (UNG) anticontamination technology. However, care must still be taken to avoid other sources of producing false positive results. They might additionally derive from human error and/or insufficient PCR amplification and detection protocols. A special case lies in the fact that PCR also amplifies DNA from dead organisms rendering a result diagnostically correct as positive, but clinically as false-positive. In PCR, as in any other diagnostic test, the risk of creating a false-negative result also exists. In such a case, the most probable source besides human error, low target or poor amplification and detection protocols is an inhibition caused by interfering substances in a patient's sample. Strategies to recognize and overcome this issue are discussed in this article. Finally a few results from quality control studies on amplification technologies in the diagnosis of infectious agents are reviewed.

Key words: Diagnostics; PCR; False-positive; False-negative; Quality control.

Abbreviations: IC internal control; PCR polymerase chain reaction; UNG uracil-N-glycosylase.

Introduction

Standardization, quality assurance and quality control are important issues in any routine diagnostic testing. They all serve to establish, maintain and guarantee a high level of quality in the performance of a laboratory to provide correct diagnoses. This may, of course, have a major impact on the management of a diseased person, since a treatment should depend on appropriate diagnosis, but also may have commercial consequences in a highly competitive environment as in the field of laboratory diagnostics, and may have legal implications in case of a possible liability.

In the past, huge efforts have been made by private and public organizations to guarantee a high quality of diagnoses. The major tools to accomplish such an ambitious goal are standardization of protocols and methods, and regular quality testing by participation of laboratories in quality tests using panels with positive and negative control specimens that are provided and supervised by scientific, public or commercial institutions.

With the advent of modern molecular biological diagnostic technologies – the best known of which are polymerase chain reaction (PCR) methods – this situation basically did not change. The challenge, however, for a laboratory that wanted to introduce and employ this new testing became even larger because of two reasons: firstly, the molecular diagnostic tests are technically demanding and normally require more expertise from the user than most of the older conventional tests and secondly, the extreme sensitivity of PCR and similar tests might cause contamination problems (in principle one single target that gets into the test will create a positive test result). Consequently, the first diagnostic results that were obtained by the new PCR technology were a disaster because of the high rate of false-positive tests created by contamination (1, 2).

Measures to Provide the Integrity of a Positive Diagnostic PCR Result

As with any other diagnostic tests, a false-positive result with PCR-based techniques may be generated by human errors (e.g. mixing of specimens or mislabelling), but also by unspecific amplification/detection and contamination.

The first source, human error, can never be completely ruled out, but in the best case it can be minimized by careful and regular education, training and supervision of personnel, all measures that can be ascribed to good laboratory practice procedures.

Unspecific amplification/detection might be a problem, particularly if non-optimal PCR protocols (e.g. temperature for primer annealing too low, long primers, high salt concentration in reaction buffer etc.) are in use (3). This is particularly true in the case of generic detection of amplicons by gel electrophoresis. This detection method is widely used because of its general applicability, but lacks specificity. This can be overcome by identification of bands in a gel by specific probes ("Southern blotting") or by probing the amplicon directly, without previous gel electrophoresis, in a liquid oligohybridization assay or on probe-coated microwell plates or on magnetic beads etc. Some of these specific detection strategies are e.g. summarized by T. White (4). Along similar lines is the development of a so-called nested PCR. This works with two primer pairs and two different amplification tests, one after the

other, for one single target, when only the final amplification product after the second PCR reaction is analyzed (5). In addition to the enhanced sensitivity of such a nested PCR (5), it was believed that such a test would also display higher specificity (four specific hybridization events, the annealing of 2 x 2 primers, must occur to obtain the final product). In reality, however, just the opposite was frequently true: nested PCR assays produced false-positive results, because this technology is very prone to contamination. Contamination indeed was the major issue when PCR was introduced into diagnostics. The principal source for contamination lies in the fact that with one amplification by PCR around 1 billion copies ("amplicons") are produced from the original target. When new clinical samples, reagents, disposables or instruments are contaminated in a subsequent round of amplification every single amplicon can give rise to another positive result, e.g. in a new test being recognized as the original target by the very same primers (in addition to this "new" contamination risk, there exist, of course, the old ones, such as contaminating an originally negative clinical sample with material from a positive specimen by careless manipulation, labelling errors etc.)

To overcome this serious problem, the following rules were set up (6, 7):

1. A PCR test should be performed in three different rooms: one for setting up the reagents ("clean area"), the second for sample preparation (all clinical materials as well as the controls should be processed there), and the third for the amplification itself and the subsequent detection of amplicons ("dirty room"). In addition, those rooms should not be adjacent to each other, but on different floors or even better in different buildings. The workflow should be always unidirectional, i.e. in the order 1 – 2 – 3 and not the other way. The personnel should change lab coats and gloves when entering a new zone. Exchange of material and instruments is not allowed. As a consequence, each room should have its own dedicated set of pipettes.

2. Since it was observed that contaminating material was also transferred by contaminated pipettes (invasion of amplicons into the instrument), pipettes had to be designed that prevented such an invasion by using a physical barrier between pipette tip and instrument. Originally, the tips had a piston that was moved up and down when pipetting with a specially designed pipettor ("positive displacement pipette"), but meanwhile "plugged" tips (tips with filter) are commonly in use, because they are cheaper and less cumbersome to use.

3. Controls are an absolute must and particular importance was given to the negative control (this consists in the most simple case of water that was "amplified", i.e. treated like a sample to be amplified). If in one run such control showed up positive, the entire assay was invalid and had to be repeated because of a contamination issue. If the problem reoccurred, this was a strong indicator of a general contamination of the laboratory. In this case, all reagents had to be freshly prepared, and the room and instruments had to be cleaned up, which was a tedious procedure because cleansing had to be done with the help of aggressive chemicals like hydrochloric acid or bleach in order to get rid of the contaminating nucleic acids.

Taking all these measures together, it was clear that the PCR technology was not at all user-friendly and in that format would never have made its way into a routine laboratory.

The break-through came with the introduction of an elegant smart biochemical anti-contamination control called "UNG" (for the key enzyme uracil-N-glycosylase)" carry-over prevention (8). UNG is also the active substance in a commercial anti-contamination product called "AmpErase®". The basic idea is to label the amplicons that are produced during a PCR amplification process so that they can be discriminated from natural target DNA, which is crucial for the recognition of a contamination of a clinical specimen with amplicons from a previous PCR run. Such labelling is done by substituting the pyrimidine base U for T during DNA amplification, which is possible when offering only deoxyuridine triphosphate, and no deoxythymidine triphosphate, to the DNA polymerase. In this case the polymerase will incorporate a "U", where originally a "T" should be. This "label" is recognized by UNG that will destroy, upon subsequent heating at alkaline pH, the U-containing (amplicon-) DNA, but not the T-containing true target DNA. Therefore a contaminated, originally true-negative, clinical specimen will turn up negative which, otherwise, would have been classified as positive. In the case of an originally positive clinical specimen the situation is more complex, because during amplification U-containing amplicons are generated that would be substrates for UNG. The reason why nevertheless a positive signal is created lies in the fact that during the elevated temperatures required for the amplification reaction, UNG is not active so that amplicons accumulate and a positive signal is obtained.

The UNG anti-contamination has worked out so efficiently that a PCR-based diagnostic test can now be carried out in a single room, provided that the laboratory is equipped with the above mentioned three zones: one for reagent preparation, one for sample preparation and one for amplification and /detection and that the other precautionary measures (dedicated coats, gloves and pipettes with plugged tips) are observed.

There is another case where PCR seems to produce a false-positive result, that has to be briefly discussed: one has to take into consideration that the target for a PCR test is not a living micro-organism (like in cell-culture techniques), but a robust DNA molecule that might be present in a clinical sample without having any clinical significance. This might be in the case of patients that have been under (successful) treatment, where the infecting micro-organism is eradicated (dead), but a PCR test will still give a positive result because its DNA might still be present, at least for some time. For example, PCR-testing is not recommended for at least two weeks after the end of treatment for *Chlamydia trachomatis* infection (9–13), since the patients might have a positive PCR test during that period of time de-

spite being cured. Actually, in such a case, the PCR result would be analytically a true-positive one, because DNA is present, but clinically this result is a false-positive.

Measures to Ensure the Integrity of a Negative Diagnostic PCR Result

As in the case of creating a false-positive result, there are again several possibilities of generating a false-negative result by amplification methods: these are once more human error, technical problems, low target concentration in a clinical specimen and the target not being recognized because of mutations and inhibition. As in the case described above with false-positives, human error can never be ruled out, but might be very difficult to prove. Technical problems can be tackled by the use of a positive run control: if this shows up negative, the entire run is invalid and must be repeated under appropriate conditions (new reagents, adjusted instruments etc). A false-negative result, because of low target concentration, can be a serious problem but has to do with the quality of the sample preparation and hence is not an objective of this article. To prove that the target was not recognized by the chosen primers is more difficult to demonstrate. The ultimate method would be sequencing of the target DNA and comparison of the sequence with the primer sequence. More easily, a second amplification system with different primers, if available (a so called "confirmation-PCR" that also helps to rule out unspecific amplification in the case of false-positive results) can elucidate such a situation. In the routine PCR-testing, however, the last issue listed above can be expected to be the most frequent one: obtaining a false-negative result is often due to non-existing or low proficiency amplification because of inhibition. The only scientifically correct way to detect, or rule out, inhibition is to monitor amplification in each individual PCR test by means of an internal control (IC) (14). An IC is a piece of DNA that is added by the user to an amplification assay and has to show up positive in every case, where amplification has taken place. Only in this case, inhibition could be ruled out. In order to fulfil this purpose, an IC must be as similar as possible to the target. For instance, it must be recognized by the same primer pair, should have the same length, and should have the same gross base composition as the target. Of course, it must have at least one discriminating feature compared to the target, so that it can be distinguished from it. The discrimination might be accomplished by a new restriction enzyme recognition site or different probing site between target and IC. In addition, the concentration of the IC in the assay should be close to the lower detection limit so that it can monitor even weak inhibitions that could cause problems in the detection of a target DNA that is also present in low concentrations. If an IC shows up negative in a particular test, there are, however, several explanations, and such a problem cannot automatically be attributed to inhibition. The possible reasons are the following:

1. There is a technical problem so that amplification cannot occur at all. This might be due to the reagents, to the thermal cycler or caused by an inappropriate sample preparation. In this case the IC serves for quality control.

2. An amplification took place but the target concentration was so high that a competition between target amplification and IC amplification occurred leading to a suppression of the latter. In this case, since the test is positive, the IC is disregarded anyway. This is, however, only true in simple, i.e. mono-target amplification. If one runs a "multiplex-PCR (co-amplification of more than one target with more than one primer pair)", results can only be obtained for the positive (predominant) target and no further information on the other targets can be deduced, since not only amplification of IC but of the other (non-dominant) targets as well might have been suppressed.

3. There is inhibition. The chemical nature of this can be very diverse: biological material present in the clinical specimen may be responsible (hemoglobin, heparin, certain hormones), detergents and solvents coming from the sample preparation (sodium dodecyl sulfate, ethanol etc.) and/or particulate matter (15, 16).

What are the strategies to overcome inhibition? The first and simplest is just a repetition of the PCR test. The rationale behind this is that, similar to target molecules, inhibitor molecules/material might be present in a low concentration and unevenly distributed. In such a case, there is a chance that a second aliquot of a clinical specimen does not display inhibition. If such procedure does not help, the second step could be a dilution of the specimen (in the order of 1:5–1:20) assuming that by these means a possible inhibitor is diluted below a harmful concentration. The drawback of this method is that target DNA will be diluted as well so that its concentration could fall below the detection limit of the test. Other authors recommend to heat, cool, freeze or simply just let the specimen stand for a certain amount of time, because they found that at least some inhibitors were very labile (17–19). A more tedious approach would be a purification of the nucleic acid to be detected, which can be done by different separation techniques. A classical method would be phenol/chloroform/ethanol lysis and precipitation. In addition to the higher workload and working with hazardous chemicals, one has to take into consideration that such a sample preparation is not quantitative, i.e. the nucleic acids are not recovered to 100%. Recently, commercial systems for purification of nucleic acids have been introduced, which can be used in this context as well. Examples are the High Pure™ product line from Roche (20) and QIAamp® from Qiagen (21).

Quality Control Testing in Amplification Diagnostics

To the author's knowledge, quality control tests by using independent "panels" have been performed and published in the following arenas:

(1) *Mycobacterium tuberculosis* (22, 23)

(2) Viral diagnosis (hepatitis C virus, human immunodeficiency virus, hepatitis B virus and cytomegalo virus) (24).

The panels consisted of real or mock positive and negative clinical specimens and in the case of viral load determinations, of specimens with dilution series of the tested virus. The amplification methods that were used for (1) above were: in house PCR methods ("home brew") and commercial amplification systems (based on transcription mediated amplification technology from GenProbe and on PCR, AMPLICOR from Roche). For (2) home brew PCR, AMPLICOR PCR and b-DNA technology (Chiron) were employed.

All studies revealed a great need for standardization and quality control, since up to 50% of the participating laboratories had unsatisfactory results according to the evaluation criteria of the supervising institutions. When home brew methods and commercial systems were compared, the mycobacteria studies did not reveal big differences. Obviously the general performance of a particular diagnostic laboratory was the more important determining factor for the outcome of the quality control testing. In other words, apparently it did not matter if a lab with a generally high quality standard used an in house or commercial test, whereas the latter did not really help another laboratory that obviously had general quality problems. However, in viral quality testing the picture was different: here there was a clear trend in favour of a commercial system (AMPLICOR), because results were generally better in labs using that technology compared with labs having in house methods (the picture for the commercial b-DNA was not as clear cut, because only very few sites applied this technology and the results were poor).

In summary the outcome was that:

– Results obtained by amplification technology are very questionable, if a laboratory is not experienced with the application of these complex diagnostic tools.

– A strict quality assurance and quality testing program is therefore absolutely mandatory.

– A laboratory that has high quality standards can be successful with home brew and commercial tests, but it appears to be easier to maintain such standards when using the latter ones (this is at least supported by data from virological testing).

This short review summarized some of the challenges a laboratory encounters when applying nucleic acid and amplification technologies to routine diagnosis. In quality control studies, several laboratories proved that those challenges can be dealt with successfully when observing some precautions outlined above. Further standardization and automation of amplification methods, together with appropriate quality control programs, will ensure the reliability and power of this technology for its application in routine diagnosis.

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