Quality Control in Diagnostic PCR

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In a PCR lab the quality control is mainly concerned with the control of errors in performance of tests and verification of test results. It includes internal and external quality control. The internal quality control includes monitoring of laboratory performance by using control material and/or repeat measurements.

Errors in sample collection, transport and nucleic acid extraction

DNA is fairly stable when it is in the cell. The cellular DNA may get degraded by DNAse derived from bacterial contamination of the sample. The sample for DNA extraction should be processed before bacteria grow in it. The sample could be collected in a sterile container. But if the sample is already contaminated then it's collection in s sterile container would not help. DNA is a very large and fragile molecule and it can also be broken by vigorous shaking during extraction. The degraded DNA is not good for PCR and this is an important reason for false negative or poor quality of results.

RNA is far more sensitive to degradation than DNA. It can easily be degraded by RNAse present in the environment especially those derived from the cells from where RNA is being extracted. Since RNA isolation procedure takes place in a strong denaturant that renders RNAse inactive, the RNA integrity is mostly at risk prior to and after the isolation. Therefore in RNA extraction the handling of sample prior to isolation and the storage of isolated RNA are very critical.

Inhibitors of PCR

Heparin, porphyrin, SDS, phenol, and proteinase-K are potent inhibitors of PCR. SDS and phenol must be completely removed from the sample in DNA extraction. The blood samples should be collected in EDTA. Heparin should not be used as anticoagulant as it inhibits PCR. Proteinase K can be inactivated by heating. PCR may also be inhibited by chocolate that might contaminate the DNA extracted from a mouthwash. The effect of inhibitors can be reduced or abolished by diluting the sample as it also dilutes the inhibitor.

Errors in PCR

There are numerous factors that may affect the efficiency of PCR. The quality of reagents, primers, buffer pH, quality and quantity of DNA and PCR tubes etc. are all important.

1. The quality of Taq polymerase is important in diagnostic work. The cheaper brands of Taq may be alright for research work but in diagnostic work these could give false positive results.
2. The primer, especially its design, is the most important determinant in specificity of PCR (Chapter 3).

3. The concentration of DNA in PCR is also important. Apart from the quality, too much or too little DNA can give false negative results.
4. While setting up PCR it is always better to premix reagents. If ten samples are to be amplified prepare reaction mix in one master tube and then transfer to individual tubes labeled 1-10. This helps in avoiding inconsistency in pipetting etc. The DNA should always be added at the end.
5. The quality of PCR tubes is often ignored. The tube should be of very thin wall that allows quick transfer of heat. The tube should fit well in the sample block. A loose fitting tube would have air around it that could interfere with heat transfer.

False positive PCR results

PCR is an extremely sensitive technique. In thirty cycles one molecule of DNA can be amplified a billion fold. The amplified product provides an ideal structure for re-amplification. It can be carried over from one sample to another and may act as target for further amplification. The carry over is a problem that is serious as well as difficult to handle.

Identifying false positives

The false positives are identified by including good quality negative, positive and non target controls (reagent blank). In diagnostic PCR it is mandatory to include at least one negative and a positive DNA control. It is also essential to include a reagent blank that contains every thing except DNA (non target control). The latter is included to exclude contamination of the reagents by extraneous DNA. If the reagent blank shows amplification of the target DNA the result of complete batch of samples becomes null and void.

Preventing false positives

1. Sample preparation, amplification and end point analysis should be physically separated. The samples may be prepared in a separate room or in a bio-safety cabinet.
2. The place should be irradiated with UV light form time to time or when contamination is suspected. UV light can destroy DNA rendering it unsuitable for further amplification. Clean lab benches and equipment periodically with freshly diluted 10% bleach solution.
3. Separate sets of pipettes may be used for the three steps. Handling of DNA before amplification is not as serious a cause of carry over from one tube to another. It is the amplified DNA that usually causes more serious problems. In a resource constrained set-up if separate pipettes for every step can not be used then at least the amplified DNA should be handled with separate pipettes clearly marked “amplified DNA only”. Barrier filter tips are also useful in preventing the carry over due to aerosol spray of the amplified products.
4. Disposable gloves should be worn to prevent carryover of amplified DNA to the sample preparation area.

5. Re-amplification of any contaminating amplified DNA from a previous PCR can be avoided if dUTP instead of dTTP is used in PCR. The dUTP containing amplified products can be rendered un-amplifiable by enzymatic degradation of uracil with UNG (Uracil DNA Glycosylase).

Handling false positives

One should take all possible precautions in preventing contamination. Once developed it is difficult to handle. To investigate errors due to contamination by extraneous DNA proceed as follows:

1. The reagents most often contaminated include those used in extraction, working solutions of primes, PCR buffer and Taq polymerase. Less often the stock solutions of PCR buffer, dNTPs and primers etc. may also be contaminated.
2. In a stepwise manner carry out the PCR with "in-use" reagents but replacing one item at a time from the fresh stock.
3. Discard the contaminated reagent if, by luck, one is able to find it!
4. In a busy diagnostic lab there may not be enough time to investigate the source of contamination. In that case the best choice would be to discard all remaining quantities of the working reagents and prepare fresh from the stocks. Use fresh vial of Taq polymerase. In the mean while de-contaminate the work area with UV light for 30-60 minutes. The pipettes are the usual source of carry over and should be thoroughly irradiated with UV light.

False negatives

The false negatives are also serious problems that often do not get enough attention. These are best detected by inclusion of good quality positive, negative and internal controls. Inclusion of an internal PCR control is always good to reduce the risk of false negatives. This is usually done by including a separate pair of primers in the reaction tube to amplify an unrelated part of the DNA. A PCR result that shows positive internal control but no amplification of the target DNA is called a true negative.

The false negatives may be caused by any of the following:

1. Degraded, too little or too much DNA.
2. Poor quality of reagents.
3. Failure to add any constituent of reaction e.g. primer, Taq polymerase or DNA.
4. Problem with the PCR machine.

Avoiding false negatives

1. Observe precautions in extraction of DNA/RNA.
2. Use only the prescribed amount of DNA/RNA.

3. In diagnostic work only good quality of reagents should be used that are not expired. In resource constrained labs reagents may be used beyond expiry if their potency is proven by previous runs or in positive controls.
4. The reagents like PCR buffers and primers etc. should be stored in aliquots. This helps in reducing loss of quality due to repeat freeze thawing. Moreover if an aliquot of reagents is contaminated it can be conveniently discarded.
5. Wearing disposable gloves helps in preventing DNAses present on the skin from degrading DNA.
6. The PCR mix contains several ingredients whose quality and quantity can be detrimental. For example insufficient amount of Mg^{++} can result in poor amplification. Increasing the concentration of Mg^{++} can increase amplification but too much of Mg^{++} can result in false positives. Similarly poor quality of dNTPs, primers, and Taq polymerase can also cause false negatives.

Instrument calibration

Instrument calibration gets least attention in a PCR lab. Thermal cyclers like any other lab equipment require calibration from time to time. The commonest error is the difference in the temperature of the block/reaction tube and that displayed on the screen. If the block temperature is lower than the displayed temperature primers can anneal non-specifically. The result would be non-specific amplification. Higher block temperature than the displayed would result in higher denaturation and annealing temperatures. Former could rapidly denature Taq polymerase while the latter would result in difficulties in primer annealing. The net result is reduction in the amplification.

The thermal cyclers should be periodically checked for the block and the displayed temperatures. Good quality digital temperature meters should be kept in a PCR lab. Use the following procedure to check the temperature in the reaction tube:

1. Place five PCR tubes in the sample wells of the heating block (four tubes in each corner well and the fifth tube in the centre well).
2. Add 50µl water to each of the tubes and keep their lids open.
3. Turn on the thermal cycler and set to hold temperature for five minutes at 65^{o}C.
4. Once the temperature has reached 65^{o}C allow one minute for temperature of water in the tubes to reach 65^{o}C.
5. One by one measure the temperature in each of the five tubes by dipping the sensor in to the water in each tube.
6. The temperature in all of the tubes should be equal and at 65^{o}C.
7. The machine needs calibration if there is any difference of temperature between the tubes and that displayed on the screen. Calibration is done by entering the new calibration factor in the software if this right is given to the user. Otherwise an engineer's help would be required. If none of the options are available one could

use temperature settings in the thermal cycling programme making "+" or "-" adjustment for the difference in temperatures of the tubes and the display.

8. If there is any difference in temperature of individual tubes the machine needs servicing by a qualified engineer.

Bibliography

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