How To Guide

PCR Troubleshooting

Carl Robinson, PhD

The polymerase chain reaction (PCR) is among the most widely used techniques in the molecular biology lab. Enabling the amplification of specific small DNA fragments, its principle is simple but effective. Typically, performing PCR does not require high levels of training and skill or exorbitantly expensive equipment to achieve good results relatively quickly. Yet the information that can be obtained and its utility as a tool make it a powerful technique. Consequently, PCR has found a place in many areas of science, from diagnostic testing to genetic engineering and forensic analyses.

A PCR experiment that works is undoubtedly a wonderful thing. However, reaching that goal – especially if starting assay development from scratch – is not always a straightforward process and can require patience, perseverance and some trial and error. In this guide, we will explore some of the ways to pinpoint and overcome problems with your PCR experiments and achieve PCR satisfaction.

Rubbish in, rubbish out

The first port of call in troubleshooting a PCR experiment is your sample itself. For most PCR experiments, your starting material will be purified DNA or cDNA. Without good starting material, no matter what other optimizations or alterations you make to your reagents or protocol, they will likely return unsatisfactory results. Both the concentration and purity of your sample are important. These parameters can be checked using techniques such as UV/Vis spectroscopy and fluorescence assays. Running some of your sample on an electrophoresis gel can give an idea of the sample concentration according to the intensity of the band and may also reveal if the DNA in the sample has degraded.

If struggling with an assay, it is worth using a DNA sample that you know works with an established PCR assay as a positive control to rule out DNA concentration and purity, including the presence of potential reaction inhibitors, as the source of the issue.

A primer match made in heaven

So, you have good starting material. The next point to consider is the suitability of the PCR primers. The optimal length for a standard PCR primer is between 18 and 30 base pairs with a roughly 50% GC-content. However, there is some flexibility to hopefully enable a compatible primer pair to be chosen. As the primers must work together in the same reaction, it is important that they have similar melting temperatures to enable PCR cycle optimization. Melting temperatures are impacted by factors such as primer length and GC-content. While complimentary A and T bases in double stranded DNA are joined by two bonds, G and C bases are joined by three. Therefore, primer sequences containing a higher proportion of G and C bases will have higher melting temperatures as more energy is required to break the increased number of bonds.

Equally, increasing the length of a primer will also increase its melting temperature for the same reason. Additionally, to promote binding, it is desirable for the 3'end of the primer to be a C or a G.

As well as impacting melting temperatures, the sequence of primers can impact their efficiency. It is best to avoid the inclusion of sequences prone to dimerization and secondary structures. Dimers occur if regions of complementary bases are included which can lead to primers hybridizing to one another and being amplified in competition with the desired target. Secondary structures within a primer can also occur when complimentary regions are included (e.g., CCC and GGG) resulting in loops, for example, and preventing the primer from binding the target DNA.

With some PCR assays there is more flexibility in deciding on the locations for your primers. When designing a PCR reaction to generate fragments for cloning for example, there may be very little choice when it comes to the external end locations for the primers. Therefore, adjustments of length can be made to make the best of what you have to work with. However, if there is more flexibility, for example when designing a diagnostic assay, the exact regions used to site the primers can be adjusted to select sites that achieve a more optimal result. There are a host of online tools available which can assist you in optimizing your primer locations and properties.

Do also remember to double check primer sequence before ordering and once again on the vial received against the reference sequence from which you designed your primer, a mistake may mean your primer binds poorly or not at all.

Are you using the most appropriate polymerase for your application?

We've got as far as the PCR reaction itself, now what? There are a wide range of DNA polymerases available and it can be difficult to decide which to use. It is important to consider your needs and downstream applications at this point. Do you need high sequence fidelity in the amplicons generated? What size amplicon are you attempting to amplify? Will your reaction be sitting around for long periods of time prior to amplification?

Some DNA polymerases are known as "proofreaders". These are enzymes that "check their work" as they incorporate bases into the growing amplicon string. They possess 3'-to-5' exonuclease activity, enabling them to remove erroneously added bases during the amplification process. While this may be unimportant if all you need to know is if your target region is present or not, for applications like cloning – where the sequence must be exact – this type of functionality can save a lot of downstream headaches when checking your final construct only to discover errors in the sequence.

The size of the fragment you are attempting to amplify will likely be guided by your downstream intentions. For example, if destined for techniques such as capillary sequencing, it will impose its own limits on the length of fragment that is targeted, with 200 bp being typical in this case. If cloning, for example, much longer fragments (often around 1–2 kb but can be considerably longer) may be the order of the day. Different polymerases have different optimal amplicon lengths, so it is important to pick an appropriate one. This information can normally be found on vendor sites and in the technical information provided for polymerases.

If there is often likely to be a lag between reaction set-up and thermal cycling, it may be worth considering a hot start polymerase. These polymerases are inactive at ambient temperatures, often including an extended heating step prior to cycling to activate the enzyme. This prevents non-specific DNA amplification that may otherwise occur at room temperature or sometimes even lower, that may hinder your desired reaction or reduce efficiency.

Maintaining the status quo

If, after all this, your reaction is still not playing ball, we still have some more tricks to try so do not give up just yet! Most polymerases will be supplied with their own buffer. However, for problematic assays, buffer optimization kits are also available. These include a range of buffer concentration and compositions that can be substituted for the standard vendor-supplied buffer. It is best to run identical assays side by side, varying only the buffer so results can be easily compared.

Shut that lid!

This may seem obvious, but whether you are using PCR tubes, strips or plates, clip lids, sticky film or re-usable sealing mats, ensure your tubes are adequately sealed. Given the temperatures repeatedly reached during PCR cycling, failure to do so can result in sample loss. There is also an increased chance of cross-contamination between neighboring reactions, making your other reactions unreliable too.

Crank up the heat

It is likely that the polymerase you have chosen will come with guidelines for the required PCR cycling conditions for that enzyme. However, the most suitable temperature for the annealing step will be determined by the melting temperatures of the primers you are using as discussed. This will typically fall within the range of 50–65 °C. Many PCR machines are broken up into zones, enabling the temperature of the annealing step to be varied across the heating block. This means that by setting up multiple identical reactions, a range of annealing temperatures can be tested in a single experiment and the results compared to find the best fit.

Back to basics

Sometimes the most obvious solutions really are the answer, so it is important to consider the basics when troubleshooting PCR experiments – your equipment and your reagents.

Is your equipment up to date with servicing and calibration? If the PCR machine is not achieving the target temperatures, for example, it can prevent amplification from occurring. Equally, if your pipette is not accurate, even small variations in the proportions of reagents can upset the reaction balance leading to PCR failure. Thinking about your reagents, are they in date? Is it possible they could be contaminated? When having problems with a PCR it is always a good idea to start with fresh aliquots of reagents to rule out the possibility of contamination.