PCR OPTIMIZATION AND TROUBLESHOOTING

Amplification of each DNA fragment can occur only under the defined conditions which are provided by a reaction mixture. If no positive PCR result can be obtained, then the optimization of the PCR parameters must be carried out. The optimization aims to determine what the optimal concentration of each component of the reaction mixture must be in order to guarantee specific and efficient amplification.

DNA template
For efficient amplification, optimal DNA amounts should be used. Optimal DNA ranges for the PCR reaction are: 0.001 – 1 ng of plasmid DNA or 1 – 10 ng of genomic DNA. Too much DNA may result in amplification of unspecific products. Particular care should be taken when choosing a DNA isolation method. Traces of reagents derived from DNA isolation process, such as phenol, Proteinase K, or EDTA, may inhibit a PCR reaction. A good solution for this kind of inhibition problem is to dilute the DNA sample, which gives rise not only DNA dilution, but to an inhibiting agent as well.

Mg2+ concentration
Magnesium ions are cofactors in polymerase activity. They also interact with other components in the reaction mixture, form complexes with dNTPs and stabilize dsDNA, increasing its melting temperature. Each new PCR experiment therefore requires the optimization of the magnesium salt concentration. Too low the Mg2+ concentration may result in low amplification efficiency and too high concentration, in the appearance of
unspecific products. Low Mg2+ concentrations are beneficial when it is necessary to minimize a ‘background’. The recommended final concentration of Mg2+ is 1 – 4 mM, however the optimal final concentration should be determined experimentally. If a DNA sample contains EDTA or other chelating agents, then the Mg2+ concentration should be increased as appropriate.

**PCR reaction buffer**

PCR reaction buffers are designed to provide buffering pH conditions and salt concentration for optimal polymerase activity. A PCR reaction buffer usually contains:

→ Tris-HCl buffer, a dipolar ionic buffer that maintains the right pH value for DNA polymerase, which falls within the 8.3 – 9.0 range. Increasing the pH may be beneficial in certain PCR systems for the stabilization of a template and improvement of the reaction.

→ KCl salt, which facilitates primer hybridization to a DNA template; however at high concentrations, it may lead to unspecific products owing to the increasing stability of uncomplementary primer-template structures. In some cases, high KCl concentration favours amplification of small PCR products, while low KCl concentration favours amplification of larger products. A higher KCl concentration decreases the ability of large DNA fragments to denaturate, so amplification of small products is preferable.

→ Additional components, such as non-ionic detergents (e.g. Triton X-100), BSA and (NH₄)₂SO₄, which increase amplification efficiency by stabilizing the DNA polymerase.
**dNTPs concentration**
Deoxyribonucleosides triphosphates (deoxyribonucleotides) are substrates for PCR reaction; they enable the synthesis of new DNA molecules. The optimal dNTPs concentration in a reaction mixture is usually 800 μM (200 μM of each dNTP). It is vital to maintain the equimolar concentration of each dNTP. An inappropriate concentration of even one dNTP can cause the polymerase to incorporate the wrong dNTP into the newly synthetized DNA strand; in other words, it affects the fidelity of the polymerase. The optimal dNTPs concentration depends on the length of the product being amplified, the Mg2+ and the primer concentration.

**DNA polymerase**
DNA polymerase activity is one of the most important parameters for optimizing a PCR reaction. It catalyses the reaction of the DNA synthesis in the 5’→3’ direction. The final activity of polymerase usual recommended is 0.5 – 2.5 U in a 50 μl PCR reaction. Too much polymerase may cause a large number of unspecific amplification products giving a specific image of background, referred to as ‘smear’. However, if there are PCR inhibitors in the reaction, using higher DNA polymerase concentrations (2.5 – 3.0 U in 50 μl) may be necessary in order to obtain satisfactory amplification efficiency. Many types of polymerases isolated from thermophylic microorganisms are commercially available at present. They differ in some features and parameters such as fidelity, processivity, or 3’ → 5’ or 5’ →3’ exonuclease activities. This diversity makes it possible to select the right polymerase for a particular application.
The 3’ → 5’ exonuclease activity, known as proofreading activity, checks for mistakes in incorporating dNTPs into the strand being synthesized and corrects them. This activity confers accuracy on a polymerase and assures the fidelity of the DNA strand amplification.
There are also polymerases containing red dye. Use of the TaqNova-RED and Hypernova-RED DNA Polymerases decrease the risk of making a mistake during a reaction set-up e.g.
skipping the polymerase, inaccurate reagents mixing. Additionally a PCR product can be applied directly to a gel after amplification without need of mixing with a loading buffer. It saves time of performing agarose or polyacrylamide electrophoresis.

The TaqNova-RED DNA Polymerase is a 94 kDa recombinant, thermostable Taq DNA polymerase isolated from Thermus aquaticus with an addition of an inert red dye, which facilitates accurate low volume pipetting and is an indicator of an enzyme addition. The dye has no adverse effect on the outcome of PCR; yields are the same as with standard TaqNova DNA Polymerase. The thermostable TaqNova-RED DNA Polymerase catalyses DNA synthesis in a 5’->3’ direction, shows no 3’->5’ exonuclease activity, but has a 5’->3’ exonuclease activity.

Hypernova-RED DNA polymerase isolated from Pyrococcus woesei. Hypernova-RED DNA polymerase catalyses a DNA replication reaction at 72°C. The halftime of the polymerase at 95°C is over 8 hours. It has 3’->5’ exonuclease activity (proofreading activity). No 5’->3’ exonuclease activity increases stability of the PCR products. It leaves blunt-ended 3’ endings (important at molecular cloning). It allows obtaining PCR products in a very wide size range up to 10 kbp. The polymerase is recommended for the multiplex PCR as it works well in a wide range of Mg2+, salt concentration and pH. It is also recommended for the amplification of difficult templates (regions abundant in GC, palindromes and multiple repeats).

A convenient way of preparing the reaction mixtures for PCR reaction is use Master Mix. In order to set up the PCR reaction, all that needs adding to the master mix is the template, the primer set and water.
**Primers**

The designing of primer sequences has a pivotal role in obtaining satisfactory results from a PCR reaction. Useful primer parameter values depend on the application and fall within the following ranges: primer length 14 – 40 nt and GC contents 40 – 75%. When designing primers for PCR, the following aspects should be taken into consideration:

→ primers must be complementary to the inner sequence of an amplified DNA fragment;

→ the primer’s 3’-end should have sequences for degenerate codons;

→ the 3’-ends of primers should not be complementary to one another, since this presents a risk of primer-dimer formation and, therefore, of the loss of primers available for PCR amplification;

→ the 3’-ends of primers should not include palindromic sequences, which may form ‘hairpin’ structures;

→ primers should not form secondary structures;

→ uneven distribution of GC and AT content should be avoided;

→ the primer’s 3’-end should contain GC pairs, which assures better primer hybridization to the DNA template; stronger primer hybridization should occur at the 5’-end than at the 3’-end, as a strong 3’-end bond may result in strong hybridization at not entirely complementary regions and lead to amplification of unspecific products;

→ the primer’s 3’-end should not contain three or more tandem repeats of C or G, since this may lead to amplification of unspecific products owing to the increased stability of an uncomplementary bond;

→ primers with a length of 15 – 30 nt are usually used; longer primers confer greater PCR specificity, although secondary structures can be formed more easily.
PCR cycling conditions

Besides the reaction mixture formulation, the optimization of PCR cycling conditions is also necessary. The following cycling conditions are recommended:

<table>
<thead>
<tr>
<th>Stage</th>
<th>Temp. [°C]</th>
<th>Time [Min.]</th>
<th>Cycles</th>
<th>Principle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>93 – 95</td>
<td>0.5 – 4</td>
<td></td>
<td>Preliminary separation of DNA strands; the time depends on the GC content</td>
</tr>
<tr>
<td>Denaturation</td>
<td>93 – 95</td>
<td>0.5 – 4</td>
<td>25 – 40</td>
<td>Obtaining single strand DNA templates; this depends on the GC content</td>
</tr>
<tr>
<td>Primer annealing</td>
<td>Ta = 37 – 72</td>
<td>0.5 – 2</td>
<td></td>
<td>Hybridization of primers to complementary regions in a DNA template; Ta (primer annealing temperature) depends on the primer sequence</td>
</tr>
<tr>
<td>Elongation</td>
<td>68 – 75</td>
<td>0.5 – 1 (1/1000 bp)</td>
<td>5 – 10</td>
<td>Primer elongation from the 3'-end by incorporation of the dNTPs by the DNA polymerase</td>
</tr>
<tr>
<td>Final elongation</td>
<td>72</td>
<td>1 – 5</td>
<td></td>
<td>Final elongation of the products which have been amplified</td>
</tr>
</tbody>
</table>

The most critical cycle parameter is the annealing temperature of the primers (Ta). The optimal annealing temperature is usually 5°C lower than the melting temperature (Tm) of the primer-template duplex. The annealing temperature depends on the primer length and sequence. An efficient primer annealing level in initial PCR cycles is primarily determined by the copy number of DNA template in the reaction and the effective time it takes the primers to search through the template for complementary sequences. If the Ta is too high, it is impossible for the primers to bond effectively with the template. If the Ta is too low, unspecific primer binding to a template may occur. At a lower temperature, the primers may hybridize to numerous regions in a genome, which may present only partial complementarity to the primers. In most cases, the optimal annealing temperature needs to be determined experimentally by carrying out a PCR reaction at a gradient of the annealing temperature.