Factors affecting PCR

The sequences of the primers are critical to the success of the experiment, as are the precise temperatures used in the heating and cooling stages of the reaction cycle.

• Designing the oligonucleotide primers for a PCR

The primers are the key to the success or failure of a PCR experiment. If the primers are designed correctly the experiment results in amplification of a single DNA fragment, corresponding to the target region of the template molecule. If the primers are incorrectly designed the experiment will fail, possibly because no amplification occurs, or possibly because the wrong fragment, or more than one fragment, is amplified (Figure 9.4).

Working out appropriate sequences for the primers is not a problem: they must correspond with the sequences flanking the target region on the template molecule. Each primer must, of course, be complementary (not identical) to its template strand in order for hybridization to occur, and the 3’ ends of the hybridized primers should point towards one another (Figure 9.5).
The DNA fragment to be amplified should not be greater than about 3 kb in length and ideally less than 1 kb. Fragments up to 10 kb can be amplified using standard PCR techniques, but the longer the fragment the less efficient is the amplification and the more difficult it is to obtain consistent results. The amplification of very long fragments - up to 40 kb - is possible, but this requires special methods.
• The first important issue to address is the length of the primers. If the primers are too short they might hybridize to non-target sites and give undesired amplification products. To illustrate this point, imagine that total human DNA is used in a PCR experiment with a pair of primers eight nucleotides in length. The likely result is that a number of different fragments will be amplified. This is because attachment sites for these primers are expected to occur, on average, once every \(4^8 = 65,536\) bp, giving approximately 49,000 possible sites in the 3,200,000 kb of nucleotide sequence that makes up the human genome. This means that it would be very unlikely that a pair of 8-mer primers would give a single, specific amplification product with human DNA (Figure 9.6a).

![Diagram of a PCR experiment](image.png)

**Figure 9.5**
A pair of primers designed to amplify the human \(\alpha\)-globin gene. The exons of the gene are shown as red boxes, the introns as grey boxes.
What if the 17-mer primers shown in Figure 9.5 are used? The expected frequency of a 17-mer sequence is once every $4^{17} = 17,179,869,184$ bp. This figure is more than fivefold greater than the length of the human genome, so a 17-mer primer would be expected to have just one hybridization site in total human DNA. A pair of 17-mer primers should therefore give a single, specific amplification product (Figure 9.6b).

**Figure 9.6**
The lengths of the primers are critical for the specificity of the PCR.
• **Working out the correct temperatures to use**

During each cycle of a PCR, the reaction mixture is transferred between three temperatures (Figure 9.7):

• The denaturation temperature, usually 94 °C, which breaks the base pairs and releases single-stranded DNA to act as templates in the next round of DNA synthesis.

• The hybridization or annealing temperature, at which the primers attach to the templates.

• The extension temperature, at which DNA synthesis occurs. This is usually set at 74 °C, just below the optimum for *Taq* polymerase.

The annealing temperature is the important one because, again, this can affect the specificity of the reaction. DNA-DNA hybridization is a temperature-dependent phenomenon. If the temperature is too high no hybridization takes place, and the primers and templates remain dissociated (Figure 9.8a).
Figure 9.7
A typical temperature profile for a PCR.
(a) Annealing temperature is too high

Primers and templates remain dissociated

(b) Annealing temperature is too low

Mismatched hybrid – not all the correct base pairs have formed

(c) Correct annealing temperature

Priming occurs only at the desired target sites

Figure 9.8
Temperature has an important effect on the hybridization of the primers to the template DNA.
The ideal annealing temperature must be low enough to enable hybridization between primer and template, but high enough to prevent mismatched hybrids from forming (Figure 9.8c). This temperature can be estimated by determining the **melting temperature** or \( T_m \) of the primer-template hybrid. The \( T_m \) is the temperature at which the correctly base-paired hybrid dissociates ('melts'). A temperature 1-2 °C below this should be low enough to allow the correct primer-template hybrid to form, but too high for a hybrid with a single mismatch to be stable. The \( T_m \) can be determined experimentally, but is more usually calculated from the simple formula (Figure 9.9): \( T_m = (4 \times [G + C]) + (2 \times [A + T]) \) °C

in which \([G+C]\) is the number of \(G\) and \(C\) nucleotides in the primer sequence, and \([A+T]\) is the number of \(A\) and \(T\) nucleotides.

**Primer sequence:** 5’ AGACTCAGAGAGAACC 3’

4 Gs 5 Cs 7 As 1 T

![Figure 9.9](image)

Calculating the \( T_m \) of a primer.

\[
T_m = (4 \times 9) + (2 \times 8) \\
= 36 + 16 \\
= 52°C
\]
The annealing temperature for a PCR experiment is therefore determined by calculating the $T_m$ for each primer and using a temperature of 1-2 °C below this figure. Note that this means the two primers should be designed so that they have identical $T_m$s. If this is not the case, the appropriate annealing temperature for one primer may be too high or too low for the other member of the pair.