Optimal primer sequences and appropriate primer concentrations are essential for maximal specificity and efficiency in PCR. In this study, we demonstrate the effects of primer sequence and concentration on PCR performance and present general guidelines to overcome some of the problems related to primer design.

5.1 Primer sequence

The 3'-terminal sequence of the primer molecule is critical for the specificity and sensitivity of PCR. A run of 3 or more G or C bases at this position should be avoided since it may stabilize nonspecific annealing of the primer. Furthermore, a thymidine at the 3' end is not recommended, since it is more prone to mis-priming than other nucleotides (1). Most importantly, primer pairs should be checked for complementarity. Complementarity between primer sequences at the 3' end often leads to primer–dimer formation. The creation and subsequent amplification of these primer–dimers reduces the availability of primer to the template molecule resulting in decreased sensitivity or even failure of the PCR (**Figure 1**). In the illustrated example the PCR is notably less susceptible to primer–dimer formation using QIAGEN PCR Buffer rather than the PCR buffer from Supplier A which contains K^+ as the only monovalent cation.

If mutations are to be introduced via the primer into the PCR product, it is important to leave at least three bases at the 3' end of the primer which are homologous to the template DNA. Mismatches at these sites will greatly reduce the efficiency of PCR (1, 2). Therefore, when using degenerate primers, it is especially important that the 3' sequence of the primer should correspond to highly conserved regions of the target sequence. Bases located at the 5' end of the primer are less critical for primer annealing. Therefore, including recognition sites for restriction enzymes or promoter elements into the primer molecule can be easily achieved by adding these sequence elements to the 5' end. Usually, such noncomplementary sequences do not change the annealing behavior of the sequence-specific part of the primer.

Primer sequences should also be checked for self-complementarity which could introduce secondary structures like hairpin loops into the primer. Commercially available computer software can be used to search for such complementary sequences.

Effect of Primer–Dimer Formation

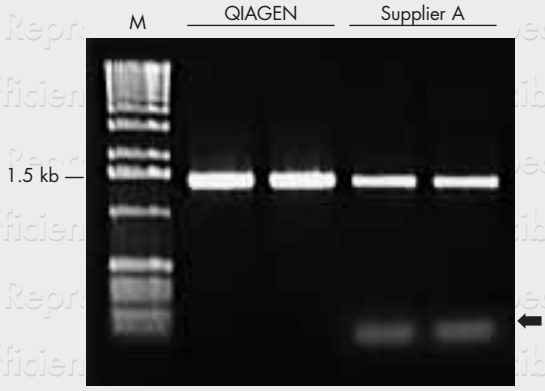

Figure 1

*Primers with a 4-bp overlap at the 3'-terminal position were employed to amplify a 1.5-kb fragment of the human single-copy cystic fibrosis gene using QIAGEN Taq DNA Polymerase and PCR Buffer (**QIAGEN**). The same PCR was performed in parallel using PCR buffer and Taq DNA polymerase from another supplier (**Supplier A**). Equal volumes of PCR product were analyzed on a 1% agarose gel. Arrow indicates primer–dimer product. **M**: markers.*

5.2 Primer length

Usually, a primer length of 18–30 bases is optimal for most PCR applications. This is based on the complexity of the target template DNA. Theoretically, a primer of 18 bases represents a unique DNA sequence amongst $4^{18} = 7 \times 10^{10}$ nucleotides and should hybridize at only one position in most eukaryotic genomes which consist of approximately 10^9–10^{10} base pairs. A shorter primer such as a 15mer would have a higher chance of annealing at more than one complementary site within the genome. This may lead to amplification of nonspecific PCR products. In contrast, when using the same shorter primer with a less complex DNA template such as plasmid DNA, PCR only generates the specific PCR product (**Figure 2**).

5.3 T_m and annealing temperature

The optimal annealing temperature often varies from the estimated T_m (3; **Table 2,** page 21) even when using pairs of primers with a similar T_m value. As a starting point, an annealing temperature 5°C below the T_m can be used. This is usually then adjusted to improve specificity and yield in a series of tedious optimization experiments. Using QIAGEN PCR Buffer such optimization of annealing temperature is often unnecessary. This is because QIAGEN PCR Buffer provides a wider temperature window for specific annealing than other commercially available PCR buffers (**Figure 3**).

Effect of Primer Length

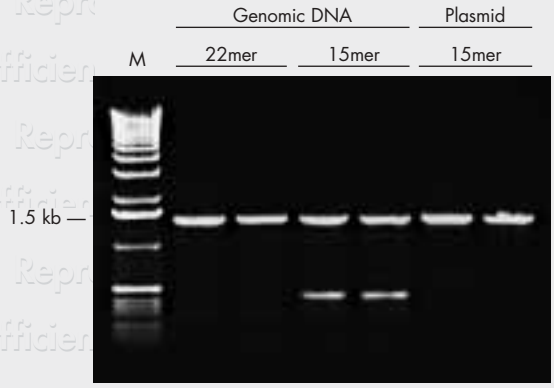

Figure 2

Amplification of a 1.5-kb fragment from the human single-copy cystic fibrosis gene, using either two 22mer primers (control) or one 22mer primer (located 5' of the fragment) and a 15mer primer (located 3' of the fragment). The 15mer primer comprised the same sequence as the 22mer control primer minus 7 nucleotides at the 3' end. PCR was performed either with human genomic DNA or with plasmid DNA containing the gene fragment to be amplified. ***M****: markers.*

Effect of PCR Buffers on Annealing Temperature

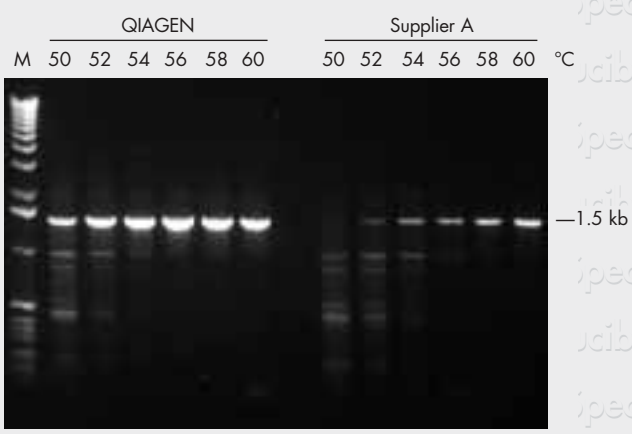

Figure 3

*Amplification of the human single-copy cystic fibrosis gene at the indicated annealing temperatures using QIAGEN PCR Buffer and QIAGEN Taq DNA Polymerase (****QIAGEN****). The same PCR was performed in parallel using PCR buffer and Taq DNA polymerase from another supplier (****Supplier A****).* ***M****: markers.*

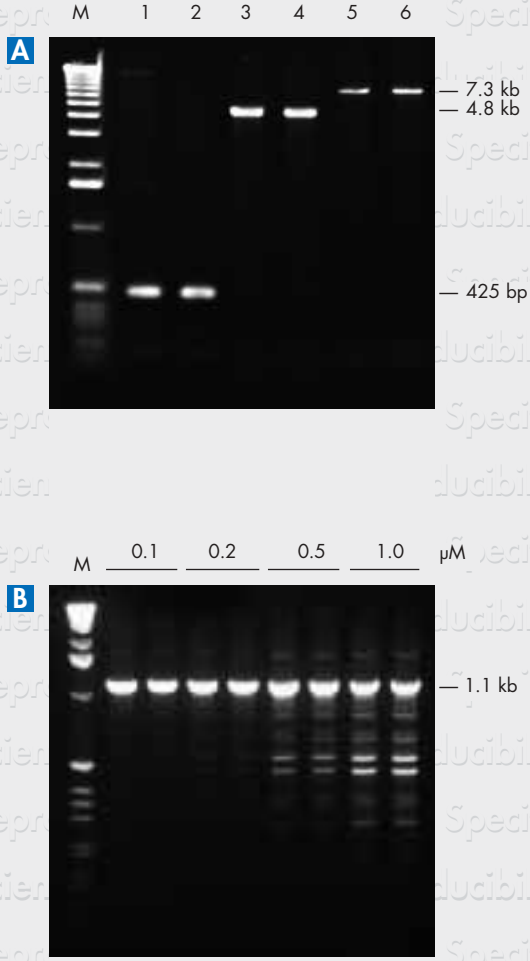

Figure 4

A *Amplification of a 425-bp fragment of the HIV* pol *gene from 10 copies of HIV DNA in 1 µg of human genomic DNA (**1, 2**), a 4.8-kb fragment of the human tissue plasminogen activator gene (**3, 4**), and a 7.3-kb fragment of the human interleukin-9-receptor gene (**5, 6**) using 0.2 µM each primer.*
B *Amplification of a 1.1-kb fragment of the human* hugl-2 *gene employing the primer concentrations indicated. **M**: markers.*

5.4 Primer concentration

The concentration of primer in the amplification reaction should be between 0.1 and 0.5 µM. For most PCR applications, including sensitive PCR assays and the amplification of longer PCR products, 0.2 µM of each primer produces satisfactory results (**Figure 4A**). Primer concentrations which are too high increase the chance of mispriming. Subsequent extension of misprimed molecules results in nonspecific PCR products (**Figure 4B**). **Table 1** gives a range of molar conversions for easy calculation of primer concentration.

Table 1.

Molar conversions for primer concentration

Primer length	pmol/µg	20 pmol*
18mer	168	119 ng
20mer	152	132 ng
25mer	121	165 ng
30mer	101	198 ng

** 20 pmol primer in a 100-µl PCR gives a primer concentration of 0.2 µM.*

Conclusions

PCR specificity and efficiency can be greatly affected by the way primers are designed and used. A summary of the guidelines for design of PCR primers is provided in **Table 2**. Even when primers are designed to have similar annealing properties, the PCR may yield nonspecific PCR products, low amounts of specific product, or fail completely. The subsequent time-consuming optimization procedures required can often be avoided when using QIAGEN *Taq* DNA Polymerase and PCR Buffer which allow for specific annealing within a wider temperature window than other PCR buffers.

Table 2.

Guidelines for the design and use of primers

Sequence:

- Avoid runs of 3 or more G or C at the 3' end
- Avoid a T at the 3' end
- Avoid mismatches at the 3' end
- Avoid complementary sequences within a primer and between primers

Length:	18–30 nucleotides
GC content:	40–60%
T_m:	T_m = 2°C x (A+T) + 4°C x (C+G)
Conc.:	0.1–0.5 µM (0.2 µM)

References

1. *Kwok, S. et al. (1990) Effects of primer–template mismatches on the polymerase chain reaction: Human immunodeficiency virus type 1 model studies. Nucleic Acids Res.* ***18****, 999.*

2. *Sommer, R. and Tautz, D. (1989) Minimal homology requirements for PCR primers. Nucleic Acids Res.* ***17****, 6749.*

3. *Suggs, S.V. et al. (1981) ICN-UCLA Symp. Mol. Cell. Biol.* ***23****, 683.*