General Notes on Primer Design in PCR
(http://www.eppendorfna.com/applications/PCR_appl_primer.asp)

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Perhaps the most critical parameter for successful PCR is the design of Primers. All things being equal, a poorly designed primer can result in a PCR reaction that will not work. The primer sequence determines several things such as the length of the product, its melting temperature and ultimately the yield. A poorly designed primer can result in little or no product due to non-specific amplification and/or primer-dimer formation, which can become competitive enough to suppress product formation. This application note is provided to give rules that should be taken into account when designing primers for PCR. More comprehensive coverage of this subject can be found elswhere[(1)].

Primer selection

Several variables must be taken into account when designing PCR Primers. Among the most critical are:

- Primer length
- Melting Temperature (T_m)
- Specificity
- Complementary Primer Sequences
- G/C content and Polypyrimidine (T, C) or polypurine (A, G) stretches
- 3'-end Sequence

Each of these critical elements will be discussed in turn.

Primer length

Since both specificity and the temperature and time of annealing are at least partly dependent on primer length, this parameter is critical for successful PCR. In general, oligonucleotides between 18 and 24 bases are extremely sequence specific, provided that the annealing temperature is optimal. Primer length is also proportional to annealing efficiency: in general, the longer the primer, the more inefficient the annealing. With fewer templates primed at each step, this can result in a significant decrease in amplified product. The primers should not be too short, however, unless the application specifically calls for it. As discussed below, the goal should be to design a primer with an annealing temperature of at least 50°C.

The relationship between annealing temperature and melting temperature is one of the "Black Boxes" of PCR. A general rule-of-thumb is to use an annealing temperature that is 5°C lower than the melting temperature. Thus, when aiming for an annealing temperature of at least 50°C, this corresponds to a primer with a calculated melting temperature(T_m) ~55°C. Often, the annealing temperature determined in this fashion will not be optimal and empirical experiments will have to be performed to determine the optimal temperature. This is most easily accomplished using a gradient thermal cycler like Eppendorf's Mastercycler® gradient.

Melting Temperature (T_m)

It is important to keep in mind that there are two primers added to a PCR reaction. Both of the oligonucleotide primers should be designed such that they have similar melting temperatures. If primers are mismatched in terms of T_m, amplification will be less efficient or may not work at all since the primer

with the higher T_m will mis-prime at lower temperatures and the primer with the lower T_m may not work at higher temperatures.

The melting temperatures of oligos are most accurately calculated using nearest neighbor thermodynamic calculations with the formula:

$T_m^{primer} = \Delta H\ [\Delta S + R \ln (c/4)] - 273.15°C + 16.6 \log_{10} [K^+]$

where H is the enthalpy and S is the entropy for helix formation, *R* is the molar gas constant and c is the concentration of primer. This is most easily accomplished using any of a number of primer design software packages on the market[3]. Fortunately, a good working approximation of this value (generally valid for oligos in the 18–24 base range) can be calculated using the formula:

$T_m = 2(A+T) + 4(G+C)$.

The table below shows calculated values for primers of various lengths using this equation, which is known as the Wallace formula, and assuming a 50% GC content[4].

Primer Length	$T_m = 2(A+T) + 4(G+C)$	Primer Length	$T_m = 2(A+T) + 4(G+C)$
4	12°C	22	66°C
6	18°C	24	72°C
8	24°C	26	78°C
10	30°C	28	84°C
12	36°C	30	90°C
14	42°C	32	96°C
16	48°C	34	102°C
18	54°C	36	108°C
20	60°C	38	114°C

The temperatures calculated using Wallace's rule are inaccurate at the extremes of this chart.

In addition to calculating the melting temperatures of the primers, care must be taken to ensure that the melting temperature of the product is low enough to ensure 100% melting at 92°C. This parameter will help ensure a more efficient PCR, but is not always necessary for successful PCR. In general, products between 100–600 base pairs are efficiently amplified in many PCR reactions. If there is doubt, the product T_m can be calculated using the formula:

$T_m = 81.5 + 16.6 (\log_{10}[K+] + 0.41 (\%G+C) - 675/length$.

Under standard PCR conditions of 50 mM KCL, this reduces to[3]:

$T_m = 59.9 + 0.41 (\%G+C) - 675/length$

Specificity

As mentioned above, primer specificity is at least partly dependent on primer length. It is evident that there are many more unique 24 base oligos than there are 15 base pair oligos. That being said, primers must be chosen so that they have a unique sequence within the template DNA that is to be amplified. A primer

designed with a highly repetitive sequence will result in a smear when amplifying genomic DNA. However, the same primer may give a single band if a single clone from a genomic library is amplified.

Because Taq Polymerase is active over a broad range of temperatures, primer extension will occur at the lower temperatures of annealing. If the temperature is too low, non-specific priming may occur which can be extended by the polymerase if there is a short homology at the 3' end. In general, a melting temperature of 55°C –72°C gives the best results (Note that this corresponds to a primer length of 18–24 bases using Wallace's rule above).

Complementary Primer Sequences

Primers need to be designed with absolutely no intra-primer homology beyond 3 base pairs. If a primer has such a region of self-homology, “snap back”, partially double-stranded structures, can occur which will interfere with annealing to the template.

Another related danger is inter-primer homology. Partial homology in the middle regions of two primers can interfere with hybridization. If the homology should occur at the 3' end of either primer, Primer dimer formation will occur which, more often than not, will prevent the formation of the desired product via competition.

G/C content and Polypyrimidine (T, C) or polypurine (A, G) stretches

The base composition of primers should be between 45% and 55% GC. The primer sequence must be chosen such that there is no PolyG or PolyC stretches that can promote non-specific annealing. Poly A and Poly T stretches are also to be avoided as these will “breath” and open up stretches of the primer-template complex. This can lower the efficiency of amplification. Polypyrimidine (T, C) and polypurine (A, G) stretches should also be avoided. Ideally the primer will have a near random mix of nucleotides, a 50% GC content and be ~20 bases long. This will put the T_m in the range of 56°C – 62°C[(1)].

3’-end Sequence

It is well established that the 3' terminal position in PCR primers is essential for the control of mis-priming[(5)]. We have already explored the problem of primer homologies occurring at these regions. Another variable to look at is the inclusion of a G or C residue at the 3' end of primers. This “GC Clamp” helps to ensure correct binding at the 3' end due to the stronger hydrogen bonding of G/C residues. It also helps to improve the efficiency of the reaction by minimizing any “breathing” that might occur.

Conclusion

It is essential that care is taken in the design of primers for PCR. Several parameters including the length of the primer, %GC content and the 3' sequence need to be optimized for successful PCR. Certain of these parameters can be easily manually optimized while others are best done with commercial computer programs. In any event, careful observance of the general rules of primer design will help ensure successful experiments.

References

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