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Primer designing for PCR

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ABSTRACT

Design your PCR primers to be 18-30 oligonucleotides in length. The longer end of this range allows higher specificity and gives you space to add restriction enzyme sites to the primer end for cloning. Make sure the melting temperatures (T_m) of the primers used are not more than 5°C different from each other. You can calculate T_m with this formula: $T_m = 4(G + C) + 2(A + T)$°C. Aim for a T_m between 52 and 58°C for each primer over the region of hybridization. Use an annealing temperature (T_a) of 3-5°C lower than the T_m. The GC content of each primer should be in the range of 40-60% for optimum PCR efficiency. Try to have uniform distribution of G and C nucleotides, as clusters of G's or C's can cause non-specific priming. Avoid long runs of the same nucleotide. Check that primers are not self-complementary or complementary to the other primer in the reaction mixture, as this will encourage formation of hairpins and primer dimers and will compete with the template for the use of primer and reagent. If you can, make the 3′ end terminate in C or A, as the 3′ is the end which extends and neither the C nor A nucleotide wobbles. This will increase the specificity. You can avoid mispriming by making the 3′ end slightly AT rich. Use the right software. Using the right software is a great way to automate these steps and minimize errors, especially when you have to design primers for many sequences.

Key words: GC content; melting temperature; nucleotides; PCR Primer; restriction enzymes.

INTRODUCTION

Polymerase chain reaction (PCR) is widely accepted as one of the most important inventions of the 20th century in molecular biology. Small amounts of the genetic material can now be amplified to be able to identify and manipulate DNA, detect infectious organisms, detect genetic variations including mutations and numerous other tasks.[1]

PCR involves three steps: denaturation, annealing and extension. First, the genetic material is denatured, converting double-stranded DNA molecules to single strands. The primers are then annealed to the complementary regions of the single-stranded molecules. In the third step, they are extended by the action of the DNA polymerase. All these steps are temperature-dependent and the common choice of temperatures is 94, 60 and 70° C, respectively.

Good primer design is essential for successful PCR. The following is a brief descrip-

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tion of the important considerations for designing a primer:

Primer length

It is generally accepted that the optimal length of PCR primers is 18-22 bp. This length is long enough for adequate specificity, and short enough for primers to bind easily to the template at the annealing temperature.[6]

Melting temperature

Melting temperature (T_m) is defined as the temperature at which one half of the DNA duplex will dissociate to become single-stranded and indicates the duplex stability. Primers with melting temperatures in the range of 52-58°C generally produce the best results. Primers with higher melting temperatures have a tendency for secondary annealing. The GC content of the sequence gives a fair indication of the T_m.[2]

Primer annealing temperature

The primer melting temperature is the estimate of the DNA-DNA hybrid stability and critical in determining the annealing temperature. Too high T_m produces insufficient primer-template hybridization resulting in low PCR product yield. Too low T_m may possibly lead to non-specific products caused by a high number of base pair mismatches. Mismatch tolerance is found to have the strongest influence on PCR specificity.[3]

GC content

The GC content (the number of guanine and cytosine in the primer as a percentage of the total bases) of primer should be 40-60%.[4]

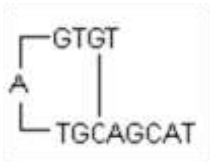
GC clamp

The presence of G or C bases within the last five bases from the 3′ end of primers (GC clamp) helps to promote specific binding at the 3′ end due to the stronger bonding of G and C bases. More than 3 G's or C's should be avoided in the last 5 bases at the 3′ end of the primer.[5]

SECONDARY STRUCTURES

Presence of the secondary structures produced by intermolecular or intra-molecular interactions can lead to poor or no yield of the product. They adversely affect primer template annealing and thus the amplification. They greatly reduce the availability of primers to the reaction.[3]

i) *Hairpins* are formed by intra-molecular interaction within the primer and should be avoided. Presence of hairpins at the 3' end most adversely affects the reaction.



ii) *Self dimer* is formed by intermolecular interactions between the two (same sense) primers, where the primer is homologous to itself. Generally a large amount of primers are used in PCR compared to the amount of target gene. When primers form intermolecular dimers much more readily than hybridizing to target DNA, they reduce the product yield.

iii) *Cross dimers* are formed by intermolecular interaction between sense and anti-sense primers, where they are homologous.

Repeats

A repeat is a di-nucleotide occurring many times consecutively and should be avoided

because they can misprime. For example: ATATATAT. A maximum number of di-nucleotide repeats acceptable is 4 di-nucleotides.

Runs

Primers with long runs of a single base should generally be avoided as they can mis-prime. For example, AGCGGGGGATG-GGG has runs of base 'G' of value 5 and 4. A maximum number of runs accepted is 4 bp.[5]

Avoid template secondary structure

A single stranded nucleic acid sequences is highly unstable and fold into conformations (secondary structures). The stability of these template secondary structures depends largely on their free energy and melting temperatures (T_m).

Avoid cross homology

To improve specificity of the primers it is necessary to avoid regions of homology. Primers designed for a sequence must not amplify other genes in the mixture. Commonly, primers are designed and then BLASTed to test the specificity.

PARAMETERS FOR PRIMER PAIR DESIGN

The amplicon length is dictated by the experimental goals. For qPCR, the target length is closer to 100 bp and for standard PCR, it is near 500 bp. If you know the positions of each primer with respect to the template, the product is calculated as: Product length = (position of antisense primer-Position of sense primer) + 1.

Product position primer can be located near the 5' end, the 3' end or any where within specified length. Generally, the sequence close to the 3' end is known with greater confidence and hence preferred most frequently.

T_m of product melting temperature (T_m) is the temperature at which one half of the DNA duplex will dissociate and become single stranded. The stability of the primer-template DNA duplex can be measured by the melting temperature (T_m).[6]

Optimum annealing temperature (T_a Opt) The formula of Rychlik is most respected. It usually results in good PCR product yield with minimum false product production.

T_a Opt = 0.3 x(T_m of primer) + 0.7 x(T_m of product) - 25

Where, T_m of primer is the melting temperature of the less stable primer-template pair T_m of product is the melting temperature of the PCR product.

Primer pair T_m mismatch calculation The two primers of a primer pair should have closely matched melting temperatures for maximizing PCR product yield. The difference of 5°C or more can lead no amplification.[6]

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