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General Concepts for PCR Primer Design

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PCR is a technology born of the modern molecular biology era. The enzyme used for PCR, *Taq* DNA polymerase, supplied with the 10× buffer, is purchased as a cloned product, and the nucleoside triphosphates are ultrapure, buffered, and available at a convenient concentration. Yet, with all of these commercially available starting materials, PCR still fails, particularly for the novice. Assuming that all of the reagents have been added in the proper concentrations, two critical PCR components are left to the researcher. The first is the nucleic acid template, which should be of sufficient quality and contain no inhibitors of *Taq* DNA polymerase (although when it comes to template purity, PCR is more permissive than many other molecular biology techniques). The second is the selection of the oligonucleotide primers. This process is often critical for the overall success of a PCR experiment, for without a functional primer set, there will be no PCR product. Although the selection of a single primer set may be trivial, the construction of primer sets for applications such as multiplex or nested PCR becomes more challenging.

The manual selection of optimal PCR oligonucleotide primer sets can be quite tedious and thus lends itself very naturally to computer analysis. The primary factors that affect the function of the oligonucleotides—their melting temperatures as well as possible homology among primers—are well-defined and straightforward tasks that are easily encoded in computer software. Once the computer has provided a small number of candidate primer sets, the task of selection can be (and still is) performed manually. In this approach, the researcher is taking advantage of the raw speed of computer calculations, trying all possible permutations of a primer's placement, length, and relation to the other primers that meet conditions specified by the user. From the thousands of combinations tested by the computer, a software program can present just those that are suitable for the needs of the experiment. Thus, the overall "quality" (as defined by the user in program parameters) of the primers selected is almost guaranteed to be better than the handful chosen and hand-tested by the research without computer assistance.

As with any tool, understanding its function will make the end product more useful. A wide range of programs have been written to perform primer selection, varying significantly in selection criteria, comprehensiveness, interactive design, and user-friendliness.[1–10] There are also commercially available specialty primer design software programs that offer enhanced user interfaces, additional features, and updated selection criteria,[1,2] as well as primer design options that have been added to larger, more general software packages.

Although most people would agree that application of analytic computer software to a well-defined problem is a smart thing to do, not all researchers are convinced that PCR primer selection is a nontrivial task, or that the selection rules that make a primer amplify efficiently are even well defined. Even though many of the rules discussed have been fine-tuned by collective empirical wisdom, most are based on firm theoretical ground, if not common sense. The purpose of this chapter is to explain basic rules of oligonucleotide primer design. With this understanding of primer selection criteria, the information deduced by primer design software can be rationally interpreted and manipulated to fit your experimental needs.

PARAMETERS USED IN BASIC PCR PRIMER DESIGN

Primer design is aimed at obtaining a balance between two goals: specificity and efficiency of amplification. *Specificity* is defined as the frequency with which a mispriming event occurs. Primers with mediocre to poor specificity tend to produce PCR products with extra unrelated and undesirable ampli-

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cons as visualized on an ethidium bromide-stained agarose gel. *Efficiency* is defined as how close a primer pair is able to amplify a product to the theoretical optimum of a twofold increase of product for each PCR cycle.

Given a target DNA sequence, analysis software attempts to strike a balance between these two goals by using preselected default values for each of the primer design variables. These variables, listed below, have predictable effects on the specificity and efficiency of amplification. Depending on the experimental requirements, these "primer search parameters" can be adjusted to override the default values that are meant to be effective for only general PCR applications. For example, in medical diagnostic PCR applications, search parameters and reaction conditions would be adjusted to increase specificity at the cost of some efficiency, because avoiding false-positive results is a higher priority in this case than producing large quantities of amplified product. By carefully considering the following parameters when using primer design software, more effective selection of primers will be achieved.

Primer Length

The specificity is generally controlled by the length of the primer and the annealing temperature of the PCR reaction. Oligonucleotides between 18 and 24 bases tend to be very sequence specific if the annealing temperature of the PCR reaction is set within a few degrees of the primer T_m (defined as the dissociation temperature of the primer/template duplex). These types of oligonucleotides work very well for standard PCR of defined targets that do not have any sequence variation. The longer the primer, the smaller the fraction of primed templates there will be in the annealing step of the amplification. In exponential amplification, even a small inefficiency at each annealing step will propagate to produce a significant decrease in amplified product. In summary, to optimize PCR, the utilization of primers of a minimal length that ensures melting temperatures of 54°C or higher will provide the best chance for maintenance of specificity and efficiency.

Short oligonucleotides of 15 bases or less are useful only for a limited amount of PCR protocols such as the use of arbitrary or random short primers in mapping simple genomes and in the subtraction library protocol described by Williams and Liang and Pardee.[11,12] Depending on the genome size of the organism, there is a minimum primer length. In general, it is best to build in a margin of specificity for safety. For each additional nucleotide, a primer becomes four times more specific; thus, the minimum primer length used in most applications is 18 nucleotides. Clearly, if purified cDNA is being used, or genomic DNA is not present, the length could be reduced because the risk of nonspecific primer/template interactions will be greatly reduced. Yet, it is generally a good idea to design primers such that the synthesized oligonucleotides can be used in a variety of experimental conditions (18- to 24-mers), and the small marginal cost of oligonucleotides with four to five additional bases makes it worth the expense.

The upper limit on primer length is somewhat less critical and has more to do with reaction efficiency. For entropic reasons, the shorter the primer, the more quickly it will anneal to target DNA and form a stable double-stranded template to which DNA polymerase can bind. In general, oligonucleotide primers 28–35 bases long are necessary when amplifying sequences where a degree of heterogeneity is expected. This has proved to be generally useful in two types of applications: (1) In amplifying sequences encoding closely related molecules, such as isoforms of a protein or family of proteins within a species, as well as in the cloning of the homologous gene from a different species,[13] and (2) in amplifying the sequences of viruses such as HIV-1, where the possibility of having a set of primers with perfect complementary

to all the templates (in this example, all HIV-1 isolates) is not expected.[14,15] In both cases, one first uses primer design software to compare all available related sequences and, in such a manner, determines the DNA region with the least amount of sequence variability. These regions serve as starting places for selecting the primers. In some instances, the researcher already knows the function of the encoded protein and the domains essential to performing that function. In these cases, comparing available sequences in the regions critical for the functional activity of the related proteins within the family will aid in defining the sequences for designing new primers. Examples include the PCR cloning of an enzyme or receptor with a similar structure and function from a related species using the available structural data. With the amino acid sequence information and the help of codon usage tables for different species, both primers, or at least one of them, could be designed around the "conserved sequence." When selecting primers to amplify DNA from a different species, sequences at the 5′- or 3′-untranslated regions of the mRNA should be avoided because they may not necessarily have high degree of homology.

The placement of the 3′ end of the primer is critical for a successful PCR reaction. If a conserved amino acid can be defined, the first 2 bases of the codon, or 3 bases in the case of an amino acid encoded by a single codon (methionine and tryptophan), can serve as the 3′ end. Perfect base-pairing between the 3′ end of the primer and the template is optimal for obtaining good results; minimal mismatch should exist within the last 5 to 6 nucleotides at the 3′ end of the primer. Attempts to compensate for the mismatches between the 3′ end of the primer and the template by lowering the annealing temperature of the reactions do not improve the results, and failure of the reaction is almost guaranteed. With this concept in mind, one should evaluate all possible strategies in the design of primers when the nucleotide sequence of the template to be amplified is not known with certainty. Cases like the one described above are routinely encountered when the researcher wishes to amplify a cDNA using information from a partial protein sequence.[13] Several approaches that include the use of degenerate oligonucleotide primers covering all possible combinations for the bases at the 3′ end of the primer in the pool, as well as the use of inosine to replace the base corresponding to the third or variable position of certain amino acid condons, have been successful for cDNA cloning and for detection of sequences with possible variations.[16] Much of this type of PCR study is empirical, and different primers may have to be synthesized to obtain the desired match.

Longer primers could also arise when extra sequence information, such as a T7 RNA polymerase-binding site, restriction sites, or GC clamp, is added to primers.[17–19] In general, the addition of unrelated sequences at the 5′ end of the primer does not alter the annealing of the sequence-specific portion of the primer. In some cases, when a significant number of bases that do not match the template sequence are added to the primer, four to five cycles of amplification can be performed at a lower annealing temperature followed by the rest of the cycles at the annealing temperature, calculated with the assumption that the sequence at the 5′ end of the primer is already incorporated into the template. Additional bases at the 5′ end of the primers are frequently added when the researcher needs to clone the PCR product. In these cases, the restriction enzyme sites of choice will be the ones that do not cut within the DNA at sites other than the primer. To ensure subcloning of the whole amplified fragment of unknown sequence as a single piece, addition of sites for enzymes that recognize 6 bases or the addition of partially overlapping recognition sites for different enzymes is recommended. An important consideration when adding restriction sites to a primer is the fact that most enzymes require two or three nonspecific extra bases 5′ to their recog-

nition sequence to cut efficiently, adding to the length of the nontemplate-specific portion of the primer.[20] Another drawback of long primer sequences is in the calculation of an accurate melting temperature necessary to establish the annealing temperature at which the PCR reaction is to be performed. For primers shorter than 20 bases, an estimate of T_m can be calculated as $T_m = 4 (G+C) + 2 (A+T)$,[21] whereas for longer primers the T_m requires the nearest-neighbor calculation, which takes into account thermodynamic parameters and is employed by most of the available computer programs for the design of PCR primers.[22,23]

The Terminal Nucleotide in the PCR Primer

Kwok and colleagues have shown that the 3′-terminal position in the primer is essential for controlling mispriming.[24] For certain applications as described above, this chance of mispriming is useful. The other issue concerning the 3′ ends of the PCR primers is the prevention of homologies within a primer pair. Care has to be taken that the primers are not complementary to each other, particularly at their 3′ ends. Complementarity between primers leads to the undesirable primer–dimer phenomena in which the PCR product obtained is the result of the amplification of the primers themselves. This sets up a competitive PCR situation between the primer–dimer product and the native template and is detrimental to the success of the amplification. In cases when multiple primer pairs are added in the same reaction (multiplex PCR), it is very important to check for possible complementarity of all the primers added in the reaction. Generally, the computer programs do not allow primer pairs with 3′-end homologies and, in conjunction with the hot start technique, the chances of formation of primer–dimer products are greatly reduced.[25]

Reasonable GC Content and T_m

PCR primers should maintain a reasonable GC content. Oligonucleotides 20 bases long with a 50% G + C content generally have T_m values in the range of 56–62°C. This provides a sufficient thermal window for efficient annealing. Within a primer pair, the GC content and T_m should be well matched. Poorly matched primer pairs can be less efficient and specific because loss of specificity arises with a lower T_m and the primer with the higher T_m has a greater chance of mispriming under these conditions. If too high a temperature is used, the primer of the pair with the lower T_m may not function at all. This matching of GC content and T_m is critical when selecting a new pair of primers from a list of already synthesized oligonucleotides within a sequence of interest for a new application. For this reason we advocate the adoption of a standardized criteria for primer selection within a laboratory. By planning ahead, it is easier to mix and match selected primers, as they will all have similar physical characteristics.

PCR Product Length and Placement within the Target Sequence

All of the computer programs provide a place for selecting a range for the length of the PCR product. In general, the length of the PCR product has an impact on the efficiency of amplification.[26] The length of a PCR product for a specific application is dependent in part on the template material. Clinical specimens prepared from fixed tissue samples tend to yield DNA that does not support the amplification of large products.[27] From pure plasmid or high-molecular-weight DNA, it is relatively straightforward to obtain products >3 kb. For the purpose of detecting a DNA sequence, generally PCR products of 150–1000 bp are produced. The specifics of the size of the desired products

often depend on the application. If the purpose is to develop a clinical assay to detect a specific DNA fragment, a small DNA amplification product of 120–300 bp may be optimal. The product should be specific and efficient to produce while containing enough information for use in a capture probe hybridization assay.[28] Products in this size range can be produced using the two-step amplification cycling method, thereby shortening the length of the amplification procedure. Other PCR approaches have different optimal product lengths. For example, for the purpose of monitoring gene expression by quantitative RNA PCR, the product must be large enough that a competitive template can be constructed and both can be easily resolved on a gel. These products generally are in the 250- to 750-bp range. Here, the issue is maximizing the efficiency of both the reverse transcriptase step and the PCR. In terms of placement of the PCR primers within a cDNA sequence, two specific points should be kept in mind. The first is to try to keep the primers and product within the coding region of the mRNA. This is the unique sequence that is responsible for the production of the protein, unlike the 3′-noncoding region that will share homologies with many different mRNAs. The second point is to try to place the primers on different exons. In so doing, the RNA-specific PCR product will be different in size from one arising from contaminating DNA. If the purpose of the PCR is to clone a specific region of a gene or cDNA, then the size of the PCR product is preselected by the application. Here, the computer program can provide information about selected primer sets that flank the desired area. In some instances, when the complete sequence is required for further experiments and the PCR product to be obtained is above the ideal length, or the template is not of the best quality, overlapping PCR fragments can be amplified by designing primers flanking unique restriction sites in the template sequence. The production of a fragment containing the entire sequence will then be obtained by cutting and pasting the amplified pieces. When approaching this kind of application it is important beforehand to think about the ideal method for cloning the PCR products and how the clone will be used in the future. For example, if one wishes to utilize restriction endonuclease sites at the end of primers as described above, it is important to be sure that these enzymes do not cut within the amplified region. Software programs can provide this information.[2]

A Simple Rule for Noncomputer-based Selection

Occasionally, the PCR primers must be selected from very defined regions at the 3′ and 5′ ends of a specific sequence. A simple method of primer design is to choose regions that are deficient in a single nucleotide. By selecting primers in this way, the chance of extensive primer-primer homology is reduced. Here, again, care must be taken to have a balanced primer pair in terms of length and base composition so that the T_m of the primers are well matched.

Nested PCR

In certain situations, there are unresolvable problems with the quantity and quality of the template to be amplified. Perhaps the actual quantity of target nucleic acid is very dilute relative the rest of the material present, or there is a limit on the purity of the starting material. In certain clinical applications, both of these problems occur simultaneously.[29] In these circumstances, one approach to synthesizing a product reliably is to develop a nested PCR assay.

Generally, the sample is first amplified for 20–30 cycles using the outer primer set; a very small aliquot of this reaction is then amplified a second time for 15–25 cycles using the inner primer set. The inner set of PCR primers

is positioned within the DNA so that the complementary sequence for the inner primer pair is present in the PCR product obtained in the first amplification reaction and available to form a template/primer complex. This has been shown to be more successful than diluting and reamplifying with the same primers.[29] The position of the inner primer set is often the determining factor in the overall structure of the nested approach and will be a factor in determining the final product size. For example, in the nested PCR detection system, adapted from the original assay of Larder et al.[30] for the amino acid 215 mutation in the HIV-1 reverse transcriptase involved in azidothymidine resistance, the 3′ end of one of the inner primers must match the mutation.[30] A control inner primer set, run in parallel, detects the wild-type sequence. In general, the product of the inner set is small, 120–270 bp. When selecting nested primer sets, special care must be given to eliminate potential primer dimers and matches between members of the inner and outer primer sets. Some of the software programs for primer design have the selection of nested primers as an option.

UTILIZING PRIMER DESIGN SOFTWARE

It is important to stress that the primer selection parameters described here are general and are not necessarily implemented in the same manner among the different primer selection software. Thus, two programs using slightly different selection algorithms will rarely, if ever, select the exact same primers, even if the basic parameters are equivalently set. These discrepancies are attributable to differences in the calculation methods and the order in which the selection criteria are applied. For example, calculating the temperature of primer/template annealing can be performed in one of several ways. The original formula of Suggs and co-workers,[21] $T_m = 2°C \times (A+T) + 4°C \times (G+C)$, is popular for its simplicity and roughly accurate prediction of oligonucleotide T_m. More recently, Rhylick et al.[26] implemented T_m prediction based on nearest-neighbor thermodynamic parameters,[22,23] which appear to be slightly more accurate. Other programs base primer annealing temperatures on formulas originally developed for DNA fragments >100 nucleotides long.[31] Thus, specifying a desired primer annealing temperature to be 60°C will produce different primers from the exact same target sequence. Further work by Rychlik and co-workers[26] produced an empirically derived equation for the optimal annealing temperature of a primer pair that depends on nearest neighbor calculations. Wu et al.[32] have also empirically derived an equation, based on primer length and GC content, to determine optimal oligonucleotide annealing temperature. These examples illustrate how something as basic as primer T_m calculation can vary among the programs.

Second, different programs attack the task of primer selection very differently, applying selection criteria to reduce the number of possible primers that the program must consider while not eliminating potentially good candidates. For example, the program by Lowe and colleagues[2] only considers primers that have a 3′-end CC, GG, CG, or GC dinucleotide, which may increase priming efficiency while allowing the user to specify a range of primer lengths. In contrast, the program by Rychlik and Rhoads[1] does not impose this requirement, but instead checks primers of a single length specified by the user. Both of these approaches eliminate potentially good primers but will, in most cases, produce an adequate number of primers that meet all of the conditions considered to be important by the investigators.

The researcher using the computer software should keep in mind that the broader the selection parameters are made, the more cases the computer must consider, significantly affecting the time required for primer searches. This is one reason that search parameters should be kept as narrow and specific

as possible when clearly dictated by experimental design. More restrictive search parameters usually result in faster searches and produce primers of greater quality. In programs that attempt more difficult selection tasks, such as choosing primers that are highly conserved across many species, or section of degenerate primers from protein sequences, the basic criteria for primer section often must be relaxed before the software finds any suitable primer pairs.

Using one of the available software programs in conjunction with the information presented here should result in the selection of a good primer set; the next task is the preparation of a good nucleic acid template.

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