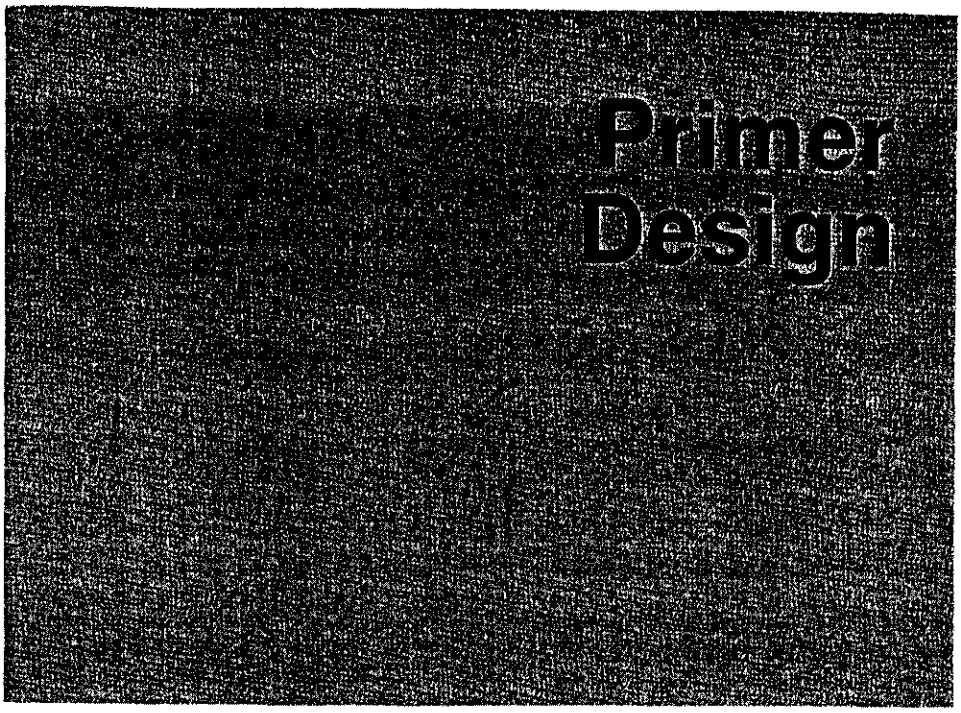

Thermostable polymerases with corresponding buffers and nucleoside triphosphates used in PCR are commercially available in ready-to-use form and at convenient concentrations. Yet, with all this prepared starting material, the amplification of certain templates can fail. Assuming that all of the reagents have been added in the proper concentrations, two critical reaction components are left to the researcher. The first is the nucleic acid template, which should be as pure as possible and should not contain DNA polymerase inhibitors (although when it comes to template purity, PCR is more permissive than many other molecular biology techniques). The second is the selection of oligonucleotide primers, often critical for the overall success of an amplification reaction.

The selection of a primer is distinctively challenging when considering applications such as multiplex PCR or nested PCR, or when designing primers based on amino acid sequences. The use of degenerate primers and base analogs, as well as considerations of primer length and codon usage in different species when designing a primer based on the amino acid sequence of a peptide, are discussed in "Design and Use of Mismatched and Degenerate Primers."

Computer-assisted primer design is more effective than manual or random selection. Some of the factors that affect the performance of primers used in PCR—melting temperatures and possible homology among primers—are well defined and can be easily encoded in computer software. The speed of computers allows calculations of all possible permutations of a primer's placement, length, and relation to other primers that meet conditions specified by the user. From the thousands of combinations tested, parameters can be adjusted so that

only those primers suitable for the needs of a particular experim
are presented. Thus, the overall "quality" (as defined by the user
the program parameters) of the primers selected by using comput
software is guaranteed to be better than those derived "manually."

As shown in the different protocols throughout this book, prime
can be designed without previous knowledge of the template
quence. These include primers that have a random nucleotide se
quence or homopolymers (i.e., oligo[dT]). Complete homology wi
the template is not required and, therefore, primers may contai
promoter elements, restriction enzyme recognition sites, or a variet
of modifications at their 5′ ends. These modifications to the primer d
nothing to hinder the PCR, but they do aid the researcher in the futur
use of the amplicon.

The challenge inherent in primer design is perhaps most eviden
in PCR using more than one primer pair, as described in "Multiple
PCR." Because more than one primer set is added to a reaction, eac
primer sequence has to be compared to the rest of the primers in th
reaction to avoid amplification failure due to primer-dimer formatio
Some of the computer programs listed in the Appendix can compar
multiple primer pairs to determine their acceptability for use in multi
plex PCR. Some primer design programs are included in sophisticate
and expensive software packages with multiple capabilities, whereas
other programs are limited to primer design.

General Concepts for PCR Primer Design

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INTRODUCTION

One critical parameter for successful amplification in a PCR is the correct design of the oligonucleotide primers. The selected primer sequences determine the size and location of the PCR product, as well as define the T_m of the amplified region, a physical parameter that has been shown to be important in product yield. Well-designed primers can help avoid the generation of background and nonspecific products as well as distinguish between cDNA or genomic templates in RNA-PCR (see Section 5). Primer design also greatly affects the yield of the product. When poorly designed primers are used, no or very little product is obtained, whereas correctly designed primers generate an amount of product close to the theoretical values of product accumulation in the exponential phase of the reaction. Optimization of reaction conditions, such as adjusting the magnesium concentration and using specific cosolvents such as dimethylsulfoxide (DMSO), formamide, and glycerol, may be required even with a good primer pair. However, primers that do not follow the basic rules of primer design may not benefit from changes in the reaction conditions. In this case, designing a new primer set might save both time and money.

Currently, there are a wide range of computer programs for performing primer selection; they vary significantly in selection criteria, comprehensiveness, interactive design, and user-friendliness (Rychlik and Rhoads 1989; Lowe et al. 1990; Pallansch et al. 1990; Lucas et al. 1991; O'Hara and Venezia 1991; Tamura et al. 1991; Griffais et al.

1991; Makarova et al. 1992; Montpetit et al. 1992; Osborne
Specialty primer design software programs that offer enhance
interfaces, additional features, and updated selection criteria (Ry
and Rhoads 1989; Lowe et al. 1990) are also available, as are pr
design options that have been added to larger, more general soft
packages. This chapter describes the basic rules for the design
good primer set.

PARAMETERS FOR BASIC PCR DESIGN

The aim of primer design is to obtain a balance between two goa
specificity of amplification and efficiency of amplification. Specifici
is the frequency with which a mispriming event occurs. Primers wi
mediocre-to-poor specificity tend to produce PCR products with extr
unrelated and undesirable amplicons, as seen on an ethidium
bromide-stained agarose gel. The efficiency of a primer is how clos
to the theoretical optimum of a twofold increase of product for each
PCR cycle a primer pair can amplify a product.

Given a target DNA sequence, primer analysis software attempts t
strike a balance between these two goals by using preselected defaul
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 applica-
tions. For example, in medical diagnostic PCR applications, search pa-
rameters and reaction conditions are adjusted to increase specificity
at the cost of some efficiency, because avoiding false-positive results
is a higher priority than producing large quantities of amplified prod-
uct. By carefully considering the following list of parameters when
using primer design software, a more effective selection of primers
will be achieved.

Primer Length

Specificity is generally controlled by the length of the primer and the annealing temperature of the PCR. Oligonucleotides between 18 and 24 bases tend to be very sequence-specific if the annealing temperature of the PCR is set within a few degrees of the primer T_m (defined as the dissociation temperature of the primer/template duplex). These 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 and result in a significant decrease in amplified product. In summary, to optimize

PCR, using primers of a minimal length that ensures melting temperatures of 54°C or higher provides the best chance for maintenance of specificity and efficiency.

Short oligonucleotides of 15 bases or less are useful only for a limited number of PCR protocols, such as the use of arbitrary or random short primers in the mapping of simple genomes and in the subtraction library protocol described by Liang and Pardee (1992) and Williams (1990). Depending on the organism's genome size, there is a bare minimum length, which will vary by a few nucleotides. 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 if genomic DNA is not present, the length can be reduced because the risk of nonspecific primer-template interactions is greatly reduced. Yet, it is generally a good idea to design primers so that the synthesized oligonucleotides can be used in a variety of experimental conditions (18- to 24-mers); the marginal cost of oligonucleotides with 4–5 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 anneals to target DNA and forms 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 primer length has proven to be generally useful in two types of applications: (1) in amplifying closely related molecules such as isoforms of a protein or family of proteins within a species, as well as in the cloning of a gene from a different species than the one whose sequence is available to the researcher (Dveksler et al. 1993); and (2) in amplifying the sequences of viruses such as HIV-1 where sequence variation and the existence of a swarm are the hallmarks of the disease and, as a consequence, the possibility of having a set of primers with perfect complementarity to all the templates (in this example, all HIV-1 isolates) is not expected (Mack and Sninsky 1988; Ou et al. 1988).

In both of these cases, one first uses the primer design software to compare all available related sequences and to describe the DNA region with the least amount of sequence variability. These regions serve as starting places to select the primers. In some instances, the researcher already knows the function of the protein and its domains essential for performing that function. In these cases, comparing available sequences in the regions critical for the functional activity of the related proteins within the family aids in the definition of the sequences around which the design of new primers should be centered.

The addition of bases at the 5′ ends of the primers is frequently observed when the researcher needs to clone the PCR product. In these cases, the restriction enzyme sites of choice are those 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, the 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 that most enzymes require 2 or 3 nonspecific extra bases 5′ to their recognition sequence to cut efficiently, thus adding to the length of the nontemplate-specific portion of the primer (New England Biolabs catalog 1993/1994, pp. 180–181). 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 is to be performed. For primers shorter than 20 bases, an estimate of T_m can be calculated as $T_m = 4(G+C)+2(A+T)$ (Suggs et al. 1981), 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 (Breslauer et al. 1986; Freier et al. 1986).

The Terminal Nucleotide in the PCR Primer

Kwok and colleagues have shown that the 3′-terminal position in the primers is essential for controlling mispriming (Kwok et al. 1990). For some of the applications described above, this chance of mispriming is useful. The other issue with the 3′ ends of the PCR primers is the prevention of homologies within a primer pair. Care must 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 phenomenon, in which the obtained PCR product 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 where multiple primer pairs are added in the same reaction (as in multiplex PCR), it is very important to double 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; thus, when they are used in conjunction with the hot start technique, the chances of formation of primer-dimer products are greatly reduced (Chou et al. 1992).

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, which provides a sufficient thermal wind for efficient annealing. The GC content and T_m should be matched within a primer pair. Poorly matched primer pairs can less efficient and specific because loss of specificity arises with lower T_m value; the primer with the higher T_m value has a grea chance of mispriming under these conditions. If too high a tempe ture is used, the primer of the pair with the lower T_m value may n function at all. This matching of GC content and T_m is critical wh selecting a new pair of primers from a list of already-synthesiz oligonucleotides within a sequence of interest for a new applicatio For this reason, we advocate the adoption of a standardized approa to primer design for the laboratory. By planning ahead, it is easier mix and match selected primers, because they will all have simil physical characteristics.

PCR Product Length and Placement within the Target Sequence

All of the computer programs provide a place to select a range for th length of the PCR product. In general, the length of the PCR produc has an impact on the efficiency of amplification (Rychlik et al. 1990). 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 amplifica tion of large products (Greer et al. 1991). It is relatively straightfor ward to obtain products greater than 3 kb from pure plasmid or high-molecular-weight DNA. For the purpose of detecting a DNA sequence PCR products of 150–1000 bp are generally 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 and also contain enough information for use in a capture probe hybridization assay (Whetsell et al. 1992). Products in this size range can be produced using the two-step amplification cycling meth-od, thereby shortening the length of the amplification procedure.

Other PCR approaches have different optimal product lengths. For example, to monitor gene expression by quantitative RNA-PCR, the product must be large enough so that a competitive template can be constructed and both the product and the competitor can be easily resolved on a gel. These products tend to run in the 250- to 750-bp range. Here the issue is to maximize the efficiency of both the reverse transcriptase step and the PCR.

In terms of placing the PCR primers within a cDNA sequence, two specific points should be kept in mind. First, try to keep the primers and product within the coding region of the mRNA, as this is the

unique sequence responsible for the production of the protein, unlike the 3′-noncoding region, which shares homologies with many different mRNAs. Second, try to place the primers on different exons so that the RNA-specific PCR product is 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 the correct primers flanking unique restriction sites in the template sequence. The production of a fragment containing the entire sequence can then be obtained by cutting and pasting the amplified pieces. When approaching this kind of application, it is important to think ahead of time about the ideal method for cloning the PCR products and how the clone will be used in the future. For example, if utilizing restriction endonuclease sites at the end of the primers as described above, it is important to be sure that these enzymes do not cut within the amplified region. Again, the software programs can provide this information (Lowe et al. 1990).

A Simple Rule for Non-computer-based Selection

Occasionally, 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 here is to choose regions that are deficient in a single nucleotide. Selecting primers in this way reduces the chance of extensive primer-primer homology. Here again, care must be taken to have a balanced primer pair in terms of length and base composition so that the T_m values of the primers are within 2–3°C of each other.

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 to the rest of the material present, or there is a limit on the purity of the starting material. Both of these problems occur simultaneously in certain clinical applications (Albert and Fenyo 1990). 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, then a very small aliquot of this reaction is amplified a second time for 15–25 cycles using the inner primer set. The inner

One example of this is the PCR cloning of an enzyme or receptor w a similar structure and function from a related species using th available structural data. With the amino acid sequence informatio and the help of codon usage tables for different species, both primers or at least one of them, can be designed around the "conserved se quence." When selecting primers to amplify DNA from a differen species, sequences at the 5′- or 3′-untranslated regions of the mRNA should be avoided because they may not necessarily have any degree of homology.

The placement of the 3′ end of the primer is critical for a successful PCR. 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 coded for by a single codon [methionine and tryptophan]) can serve as the 3′ end. Perfect base pairing between the primer's 3′ end and the template is optimal for obtaining good results, and minimal mismatches should exist within the last 5–6 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 encountered routinely when the researcher wishes to amplify a cDNA using information from a partial protein sequence (Dveksler et al. 1993). Several approaches, including the use of degenerate oligonucleotide primers that cover 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 codons, have been successful for cDNA cloning as well as for the detection of sequences with possible variations (Lin and Brown 1992). 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 a GC clamp, is added to primers (Loh et al. 1989; Sheffield et al. 1989; Kain et al. 1991). In general, the addition of unrelated sequence 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; this is 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.

set of PCR primers is positioned within the DNA so that the
plementary sequence for the inner primer pair is present in the
product obtained in the first amplification reaction and availabl
form a template-primer complex. This has been shown to be mo
successful than diluting and reamplifying with the same primers
bert and Fenyo 1990). The position of the inner primer set is often
determining factor in the overall structure of the nested approach a
is a factor in determining the final product size. For example, in
nested PCR detection system, adapted from the original assay of L
der for the amino acid 215 mutation in the HIV-1 reverse transcripta
involved in zidovudine resistance, the 3′ end of one of the inn
primers must match the mutations to produce a quality PCR produ
(Larder et al. 1991). A control inner primer set run in parallel detec
the wild-type sequence. In general, the product of the inner set
small, 120–270 bp. The outer primer pair ideally should completel
flank the inner product. When selecting nested primer sets, specia
care must be given to eliminating potential primer-dimers an
matches between members of the inner and outer primer sets. Som
of the software programs for primer design have the selection o
nested primers as an option.

USING 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 programs. Thus, two
programs using slightly different selection algorithms rarely, if ever,
select the same primers, even if the basic parameters are set equi-
valently. These discrepancies are due 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 et al. (1981), $T_m = 2°C \times (A+T) + 4°C \times (G+C)$, is popular for its
simplicity and roughly accurate prediction of oligonucleotide T_m.
More recently, Rychlik et al. (1990) implemented T_m prediction based
on nearest-neighbor thermodynamic parameters (Breslauer et al.
1986; Freier et al. 1986) that appear to be slightly more accurate.
Other programs base primer annealing temperatures on formulas
originally developed for DNA fragments over 100 nucleotides long
(McConaughy et al. 1969). Thus, specifying a desired primer anneal-
ing temperature to be 60°C will produce different primers from the
same target sequence. Further work by Rychlik et al. (1990) produced
an empirically derived equation for the optimal annealing tempera-
ture of a primer pair that depends on nearest-neighbor calculations.
Wu et al. (1991) 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 the program must consider, while not eliminating potentially good candidates. For example, the program by Lowe et al. (1990) only considers primers that have a 3′-end CC, GG, CG, or GC dinucleotide, which may increase priming efficiency, but allows the user to specify a range of primer lengths. In contrast, the program by Rychlik and Rhoads (1989) does not impose this requirement, but checks primers of a single length specified by the user. Both of these approaches eliminate potentially good primers but still, in most cases, produce an adequate number of primers that meet all the conditions considered important by the authors.

In using the computer software described, 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 they are 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 selection of degenerate primers from protein sequences, the basic criteria for primer selection often must be relaxed to find primers that satisfy more critical needs.

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

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