Chapter 4: Oligonucleotides as Primers

The use of oligos as primers for PCR and DNA sequencing account for more than 70% of the use of oligonucleotides. All enzymatic-derived DNA synthesis requires a 3' hydoxyl group from an exogenous source of either RNA or DNA. The oligo primer provides the necessary 3' hydoxyl group. The specificity of the oligo primer to base-pair to a defined region of its target also insures that the enzymatic primer directed DNA synthesis is anchored precisely and that the synthesis product is fixed at its 5'end. Without this precise anchoring afforded by the oligo primer, such techniques as enzymatic DNA sequencing or PCR would not be possible.

General considerations for the selection of primers are: a) the oligo should be 18-24 bases in length; b) the Tm should be between 50-65ºC; c) the GC content should be 40-60%; d) primer should only bind to one site of the template; e) avoid sequences that would produce internal secondary structure; and f) the primer has a free 3' hydroxyl.

PCR Primer Design

The primers for PCR determine the size of the product, the sequence location of the PCR product and the Tm of the amplified region. Well-designed primers avoid generation of background and non-specific products. We recommend that a computer-aided program be used during primer design in order to avoid certain fatal design flaws. Several commercial programs are available, as well as several internet WEB sites, whose function is to select PCR primer pairs that balance specificity of amplification and efficiency of amplification. Primer specificity is a measure of the frequency with which mispriming events occur, while primer efficiency is a measure of how close to theoretical optimum of twofold increase of product for each PCR cycle a primer can amplify a product.

PCR specificity is controlled by length and annealing temperature of PCR, with annealing temperature being dependent upon primer Tm. The following general primer pair design guidelines have proven to be successful:

- Primer pairs between 18-24 nucleotides in length tend to be target-specific.
- The primer pairs should be complementary to particular sites on the template DNA.
- Each primer should bind to only one of the two template strands.
- The optimum distance between primer annealing sites is generally 100-1000 base pairs.

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- The GC content of the primers should be between 40-60%.
- Ideally, both primers should anneal at the same temperature. At minimum, the primers should have Tm's within 5ºC of each other. The optimal annealing temperature is dependent upon the primer with the lowest melting temperature. As a rule, 20mer primer pairs with 50% GC content have Tm's between 56-62ºC.
- Primer sequence should start and end with 1-2 purine bases.
- The placement of the 3' end is critical for success. Perfect base pairing between the primer 3' end and template is optimal. If a conserved amino acid sequence can be defined, use the first two bases of the codon as 3' end.
- There should be minimal mismatch within the last 5-6 nucleotides at the 3' end.
- Avoid three G or C nucleotides in a row near the 3´-end of the primer.
- Simple rule: choose regions that are deficient in a single nucleotide.

In general, addition of unrelated sequences at the 5' end does not alter annealing of the sequence-specific portion of the primer. Many times these additions are recognition sequences for restriction enzymes. If restriction sites are to be added, include 2-3 non-specific extra bases 5' to the recognition site to allow more efficient cutting by the enzyme. If a significant number of unrelated bases is added, 4-5 cycles of amplification at a lower annealing temperature, followed by cycles with optimal annealing temperature, may be used.

Keep the primers and product sequences within the coding region of the mRNA. Place primers on different exons so RNA-specific PCR is different in size from DNA contamination.

If no primer candidates survive the above criteria, relax the stringency of the selection requirements. The best test of a good primer is only in its use, and not all working primers can be accurately predicted by the above general rules.

Degenerate PCR primers

The use of degenerate PCR has proven to be a very powerful tool to find "new" genes or gene families. Most genes come in families that share structural similarities. By aligning the protein sequences from a number of related proteins, one can find shared conserved sequences which can be used as a starting point to make degenerate PCR primers. Several general rules for the construction of degenerate pools of primers from conserved amino acid motifs are:

1. Use codon bias of the appropriate species for translation.
2. Use two blocks of conserved amino acid for primer pairs.
3. Use 4-6 amino acid sequences as bases for degenerate primers. The length of the primers should be a minimum of 20 nucleotides.
4. Avoid degenerate bases at 3' end, omit the last base of terminal codon unless the amino acid is met or trp.
5. Consider using deoxyinosine to reduce degeneracy.
6. Primers ending in T are efficiently extended even when mismatched with T, G or

C. 3' terminal mismatches A-G, G-A, C-C, and G-G reduce PCR yields 100 fold, with A-A mismatch reducing yield by 20 fold.

Automated DNA Sequencing Primer Design

When designing primers for automated sequencing, use the same general criteria used for designing PCR primers. While almost any sequence primer can be made to work, by following several general rules of primer design, your chance of generating high quality DNA sequence by using cycle sequencing protocols will be greatly enhanced.

The steps in primer design are as follows:

1. If designing a primer from a sequence chromatogram, pick an area of which you are 100% sure. Avoid designing primers using regions of poorer quality (i.e., areas beyond single peak resolution).
2. Choose a priming site that is greater than 50 bases away from the position where new sequence is needed. When targeting sequences at very specific regions, position the primer so the desired sequence falls about 80-150 nucleotides away from the primer.
3. Identify candidate primers that form stable base pairing (18-24 bases in length, 40-60% GC with Tms between 55-75ºC).
4. Discard candidate primers that demonstrate undesirable self-hybridization. Avoid primers that can form 4 or more consecutive bonds with itself, or 8 or more bonds total.
5. Verify the site-specificity of the primer. Avoid primers where alternative priming sites are present with more than 90% identity to the primary site or that match at more than seven consecutive bases at the 3'end.
6. Choose among the candidate primers that are more A-T rich at the 3'end. Thesetend to be slightly more specific in action.

Like the PCR primer design criteria, be aware that no set of guidelines will accurately predict the success of a primer. Some primers may fail for no apparent reason, and primers that appear to be poor candidates may work very well.

WEB sites for designing primers

PCR Primers

Oligos-U-Like Primer Design

http://www.path.cam.ac.uk/cgi-bin/primer3cgi

The Oligos-U-Like program allows the user to specify up to 56 different parameters. Fortunately, defaults are provided. Just enter the DNA sequence and a list of primer pairs and results including length, starting base number, Tm, %GC and PCR product size for each pair are given.

CODEHOP-PCR primer designed from protein multiple sequence alignments

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http://www.blocks.fhcrc.org/codehop.html

The CODEHOP program designs PCR primers from protein multiple-sequence alignments. The program is intended for cases where the protein sequences are distant from each other and degenerate primers are needed. The multiple-sequence alignments should be of amino acid sequences of the proteins and be in the Blocks Database format. Proper alignments can be obtained by different methods. The results of the CODEHOP program are suggested degenerate sequences of DNA primers that you can use for PCR. You have to choose appropriate primer pairs, get them synthesized and perform the PCR.

Web Primer

http://genome-www2.stanford.edu/cgi-bin/SGD/web-primer

PCR primers:

There are many factors that influence the success of a pair of primers. Some of the properties of primers which can affect the outcome of PCR include: the GC / AT ratio, length, melting temperature, and the extent of annealing between primers. The location of a primer also heavily influences its usefulness. All of these variables are able to be influenced by the user.

Sequencing Primers:

Sequencing primers are also highly customizable. Potential valid primers are evenly spaced along the DNA of interest starting at each 5' end. The user is allowed to specify the area of DNA to be sequenced, how many strands to order sequencing primers for, which strand to order primers for, the approximate distance between primers, the length and percent GC content of the primers, and the maximum self annealing of the primers.