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Minireview

Bioinformatic tools and guideline for PCR primer design

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Bioinformatics has become an essential tool not only for basic research but also for applied research in biotechnology and biomedical sciences. Optimal primer sequence and appropriate primer concentration are essential for maximal specificity and efficiency of PCR. 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. There are several online tools devoted to serving molecular biologist design effective PCR primers. This review intends to provide a guide to choosing the most efficient way to design a new specific-primer by applying current publicly available links and Web services. Also, the purpose here is to provide general recommendations for the design and use of PCR primers.

Key words: Bio-computing, primer design, web-based resources.

INTRODUCTION

In the last 10 to 15 years the computer has become an essential companion for cell and molecular biologists. Bioinformatics is an emerging scientific discipline that uses information technology to organize, analyze, and distribute biological information in order to answer complex biological questions. Bioinformatics is an interdisciplinary research area, which may be broadly defined as the interface between biological and computational sciences (Singh and Kumar, 2001). It involves the solution of complex biological problems using computational tools and systems. It also includes the collection, organization, storage and retrieval of biological information from databases. Selection of oligonucleotide primers is useful for polymerase chain reaction (PCR), oligo hybridization and DNA sequencing. Proper primer design is actually one of the most important factors/steps in successful DNA sequencing. Various bioinformatics programs are available for selection of primer pairs from a template sequence. The plethora programs for PCR primer design reflects the central role of PCR in modem molecular biology. Nevertheless, all these computer programs are written mainly to assist in the primer design process and are not meant to replace the eye of the experienced researcher, especially considering the sometimes erratic nature of PCR experiments. When scheduling important PCR experiments, it is usually worthwhile to evaluate the predictions of numerous different programs and to use common sense and laboratory experience to evaluate the suggested primers before committing to their synthesis (Binas, 2000). This review summarizes the general guidelines for primer design online.

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WEB-BASED RESOURCES FOR PRIMER DESIGN

There are a numerous web-based resources for PCR and primer design. Though most are freely available, they are of variable quality and not well maintained. This often results in missing links and so sites that may have been useful previously may not be functional at a later date. There are a number of criteria that need to be established in the design of primers and a number of these are listed below (Tables 1and 2).

SOFTWARE IN PRIMER DESIGN

The use of software in biological applications has given a new dimension to the field of bioinformatics. Many different programs for the design of primers are now available. Freeware software is available on the internet and many universities have established servers where a user can log on and perform free analyses of proteins and nucleic acid sequences (Singh and Kumar, 2001). There are number of simple stand-alone programs as well as complex integrated networked versions of the commercial software available. These software packages may be for complete DNA and protein analysis, secondary structure predictions, primer design, molecular modeling, development of cloning strategies, plasmid drawing or restriction enzyme analyses. Companies engaged in biosoftware development include: Alkami Biosystems, Molecular Biology Insights, PREMIER Biosoft International, IntelliGenetics Inc., Hitachi Inc., DNA Star, Advanced American Biotechnology and Imaging. Some scientists have also developed algorithms and computer programs for various purposes of primer design (Rychlik and Rhoades, 1989; Lowe et al., 1990; Lucas et al., 1991; O'Hara and Venezia, 1991; Tamura et

Table 1. Online primer design sites.

Tool name	Description	www
CODEHOP	**C**onsensus **D**egenerate **H**ybrid **O**ligonucleotide **P**rimers; degenerate PCR primer design; will accept unaligned sequences.	http://blocks.fhcrc.org/codehop.html
Gene Fisher	Interactive primer design tool for standard or degenerate primers; will accept unaligned sequences.	http://bibiserv.techfak.uni-bielefeld.de/genefisher/
DoPrimer	Easily design primers for PCR and DNA sequencing.	http://doprimer.interactiva.de/
Primer3	Comprehensive PCR primer and hybridization probe design tool; many options but easy to accept defaults at first.	http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi http://www.basic.nwu.edu/biotools/Primer3.html http://www.justbio.com/primer/index.php
Primer Selection	Select PCR primers from nucleotide sequence.	http://alces.med.umn.edu/rawprimer.html
Web Primer	Allow alternative design of primers for either PCR or sequencing purpose.	http://genome-www2.stanford.edu/cgi-bin/SGD/web-primer
PCR Designer	For restriction analysis of sequence mutations.	http://cedar.genetics.soton.ac.uk/public_html/primer.html
Primo Pro 3.4	Reduces PCR noise by lowering the probability of random primering.	http://www.changbioscience.com/primo/primo.html
Primo Degenerate 3.4	Primo Degenerate 3.4 designs PCR primers based on a single peptide sequence or multiple alignments of proteins or nucleotides.	http://www.changbioscience.com/primo/primod.html
PCR Primer Design	An application that designs primers for PCR or sequencing purposes.	http://pga.mgh.harvard.edu/servlet/org.mgh.proteome.Primer
The Primer Generator	The program analyzes the original nucleotide sequence and desired amino acid sequence and designs a primer that either has a new restriction enzyme site or is missing an old one.	http://www.med.jhu.edu/medcenter/primer/primer.cgi
EPRIMER3	Picks PCR primers and hybridization oligos (EMBOSS).	http://bioweb.pasteur.fr/seqanal/interfaces/eprimer3.html
PRIMO	Prediction of forward and reverse oligonucleotide Primers.	http://bioweb.pasteur.fr/seqanal/interfaces/primo.html3 http://atlas.swmed.edu/primo/primo_form.html
PrimerQuest	A primer design tool.	http://www.idtdna.com/biotools/primer_quest/primer_quest.asp
MethPrimer	Design primers for methylation PCRs.	http://itsa.ucsf.edu/~urolab/methprimer/index1.html
Rawprimer	A tool for selection of PCR primers.	http://alces.med.umn.edu/rawprimer.html
MEDUSA	A tool for automatic selection and visual assessment of PCR primer pairs.	http://www.cgr.ki.se/cgr/MEDUSA/
The Primer Prim'er Project	Software suite that completely automates the PCR primer design process.	http://www-nmr.cabm.rutgers.edu/bioinformatics/Primer_Primer_Project/Primer.html
Oligonucleotides for the PCR	Seek oligonucleotides on both sides of an area.	http://www.citi2.fr/bio2/Oligo2lib.html
GAP	Genome- wide Automated Primer finder servers.	http://promoter.ics.uci.edu/Primers/

al., 1991; Makarova et al., 1992; Osborne, 1992; Li et al.,1997; Plasterer, 1997; Sze et al., 1998; Gorelenkov et al., 2001). Many programs aiding in the design of primers exist (Table 3).

GUIDELINES FOR THE DESIGN AND USE OF PRIMERS

DNA Template and oligonucleotide primers must be considered in greater detail (Linz et al., 1990). Efficacy and sensitivity of PCR largely depend on the efficiency of primers (He et al., 1994). The ability for an oligonucleotide to serve as a primer for PCR is dependent on several factors including: a) the kinetics of association and dissociation of primer-template duplexes at the annealing and extension temperatures; b) duplex stability of mismatched nucleotides and their location; and c) the efficiency with which the polymerase can recognize and extend a mismatched duplex. The primers which are unique for the target sequence to be amplified should fulfill certain criteria such as primer length, GC%, annealing and melting temperature, 5' end stability, 3' end specificity etc (Dieffenbach et al., 1995). Most of the

Table 2. PCR oligonucleotide resources.

Oligonucléotides pour la PCR	Calculation of melting point of a oligonucleotide.	http://www.citi2.fr/bio2/OligoTM.html
Oligonucleotide properties calculator	Prediction of melting temperature.	http://www.basic.nwu.edu/biotools/oligocalc.html http://www.microbiology.adelaide.edu.au/learn/oligcalc.htm
Oligonucleotide analyzer	Generates Tm, free energy, molecular weight and hairpin and dimer formation structures.	http://www.rnature.com/oligonucleotide.html
Oligo Tm Determination	Prediction of Tm.	http://alces.med.umn.edu/rawtm.html
Poland	Prediction of melting temperatures of primers.	http://www.biophys.uni-duesseldorf.de/local/POLAND/poland.html
PROLIGO	Oligos parameter calculation.	http://www.gensetoligos.com/Calculation/calculation.html

Table 3. PCR primers design software for personal computer.

Software name	Descreption	www
PrimerSelect	Analyzes a template DNA sequence and chooses primer pairs for PCR and primers for DNA sequencing.	www.dnastar.com
DNASIS Max	DNASIS Max is a fully integrated program that includes a wide range of standard sequence analysis features.	http://www.medprobe.com/no/dnasis.html
Primer Premier 5	primer design for Windows and Power Macintosh.	http://www.premierbiosoft.com/primerdesign/primerdesign.html
Primer Premier:	Comprehensive primer design for Windows and Power Macintosh.	http://www.premierbiosoft.com/
NetPrimer	Comprehensive analysis of individual primers and primer pairs.	http://www.premierbiosoft.com/NetPrimer.html
Array Designer 2	For fast, effective design of specific oligos or PCR primer pairs for microarrays	http://www.premierbiosoft.com/dnamicroarray/dnamicroarray.html
Beacon Designer 2.1	Design molecular beacons and TaqMan probes for robust amplification and fluorescence in real time PCR.	http://www.premierbiosoft.com/molecular_beacons/taqman_molecular_beacons.html
GenomePRIDE 1.0	Primer design for DNA-arrays/chips.	http://pride.molgen.mpg.de/genomepride.html
Fast PCR	Software for Microsoft Windows has specific, ready-to-use templates for many PCR and sequencing applications: standard and long PCR, inverse PCR, degenerate PCR directly on amino acid sequence, multiplex PCR.	http://www.biocenter.helsinki.fi/bi/bare-1_html/manual.htm
OLIGO 6	Primer Analysis Software for Mac and Windows.	http://www.oligo.net/
Primer Designer 4	Will find optimal primers in target regions of DNA or protein molecules, amplify features in a molecule, or create products of a specified length.	http://www.scied.com/ses_pd5.htm
GPRIME	Software for primer design.	http://life.anu.edu.au/molecular/software/gprime.htm
Sarani Gold	Genome Oligo Designer is software for automatic large-scale design of optimal oligonucleotide probes for microarray experiments.	http://mail.strandgenomics.com/products/sarani/
PCR Help	Primer and template design and analysis	http://www.techne.com/CatMol/pcrhelp.htm
Genorama chip Design Software	Genorama Chip Design Software is complete set of programs required for genotyping chip design. The programs can also be bought separately.	http://www.asperbio.com/Chip_desin_soft.htm
Primer Designer	The Primer Designer features a powerful, yet extremely simple, real-time interface to allow the rapid identification of theoretical ideal primers for your PCR reactions.	http://genamics.com/expression/primer.htm
Primer Premier	Automatic design tools for PCR, sequencing or hybridization probes, degenerate primer design, Nested/Multiplex primer design, restriction enzyme analysis and more.	http://www.biotechniques.com/freesamples/itembtn21.html
PrimerDesign	DOS-program to choose primer for PCR or oligonucleotide probes.	http://www.chemie.unimarburg.de/%7Ebecker/pdhome.html

reviews on PCR optimization (Erlich et al., 1991; Dieffenbach et al., 1995; Roux, 1995) consider different parameters of PCR but generally do not discuss basic concepts of PCR primer design.

Maybe 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 badly 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 elsewhere (Dieffenbach et al., 1995).

The sequences of the primers used for PCR amplification can have a major effect on the specificity and sensitivity of the reaction. When choosing two PCR amplification primers, the following guidelines should be considered:

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 (Wu et al., 1991). For broad-spectrum studies, primers of typically 18-30 nucleotides in length are the best. Primers should be at least 18 nucleotides in length to minimize the chances of encountering problems with a secondary hybridization site on the vector or insert. Primers with long runs of a single base should generally be avoided. It is especially important to avoid 4 or more G's or C's in a row.

Melting Temperature (T_m): The optimal melting temperatures for primers in the range 52-58^{o}C, generally produce better results than primers with lower melting temperatures. Primers with melting temperatures above 65^{o}C should also be avoided because of potential for secondary annealing. It is then advisable to do the sequencing reaction with annealing and extension at 60^{o}C. A good working approximation of this value (generally valid for oligos in the 18–30 base range) can be calculated using the formula of Wallace et al. (1979), T_m = 2(A+T) + 4(G+C). Using improved nearest-neighbor thermodynamic values given by SantaLucia et al. (1996), an estimate of melting temperature can be obtained for oligonucleotide analysis.

GC Content (Tm and Ta are Interrelated): GC% is an important characteristic of DNA and provides information about the strength of annealing. Primers should have a GC content between 45 and 60 percent (Dieffenbach et al., 1995). For primers with a G/C content of less than 50%, it may be necessary to extend the primer sequence beyond 18 bases to keep the melting temperature above the recommended lower limit of 50^{o}C. GC content, melting temperature and annealing temperature are strictly dependent on one another (Rychlik et al., 1990).

3'-End Sequence: It is well established that the 3' terminal position in PCR primers is essential for the control of mis-priming (Kwok et al., 1990). Primers should be "stickier" on their 5' ends than on their 3' ends. A "sticky" 3' end as indicated by a high G/C content could potentially anneal at multiple sites on the template DNA. A "G" or "C" is desirable at the 3' end but the first part of this rule should apply. This GC clamp reduces spurious secondary bands (Sheffield et al., 1989).

Dimers and false priming cause misleading results: Primers should not contain complementary (palindromes) within themselves; that is, they should not form hairpins. If this state exists, a primer will fold back on itself and result in an unproductive priming event that decreases the overall signal obtained (Breslauer et al., 1986). Hairpins that form below 50^{o}C generally are not such a problem. Primers should not contain sequences of nucleotides that would allow one primer molecule to anneal to itself or to the other primer used in PCR reactions (primer dimer formation).

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. However, 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.

Degenerate Primers: Degeneracy in primer sequence should also be taken into consideration. Degenerate primers based on the amino acid sequence of conserved regions were also used to search for members of a gene family (Wilks et al., 1989). Computer programs have also been developed specifically for degenerate primer design (Chen and Zhu, 1997).

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” can occur. 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.

Other recommendations: The concentration of primer in amplification reaction should be between 0.1 and 0.5 μm. If possible, a computer search should be conducted against the vector and insert DNA sequences to verify that the primer and especially the 8-10 bases of its 3' end

are unique. Inosine should not be included in sequencing primers. They either do not work or give poor cycle sequencing results. The design of PCR and DNA sequencing primers follows very similar guidelines. Even though primer characteristics can be visually inspected for the presence of the elements listed above, a number of computer programs that have been developed use several of these guidelines for primer selection.

CONCLUSION

The key to the PCR lies in the design of the two oligonucleotide primers. 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 by hand optimized while others are best done with marketable computer programs. The increasing use of information from the internet and the sequences held in gene databases are practical starting points when designing primers and reaction conditions for the PCR. A number or software packages such as Oligo, Primer etc. have allowed the process of primer design to be less troublesome. It is also possible to include more than one set of primers in a PCR.

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