

# PCR Primer Design

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## Abstract

**To make PCR a specific, efficient and cost effective tool for researchers and clinicians the most important aspect is oligonucleotide primer design. This review discusses various aspects of primer design. Advice is provided for optimal design and the role of bioinformatic tools is highlighted. The authors discuss theoretical considerations and compare computational and experimental studies.**

## Introduction

Bioinformatics is a newly-emerged inter-disciplinary research area spanning a range of specialties that include molecular biology, biophysics, computer science, mathematics and statistics. It makes use of scientific and technological advances in the areas of computer science, information technology and communication technology to solve complex problems in life sciences, particularly problems in biotechnology. Bioinformatics comprises of the development and application of algorithms for the analysis and interpretation of data, for the design and construction of vital databases, and for the design of experiments.

Bioinformatics is used interchangeably with the terms biocomputing and computational biology. However, biocomputing is more correctly defined as the systematic development and application of computing systems and computational solution techniques to model biological phenomena. Polymerase chain reaction (PCR) is one such phenomenon. PCR is used for the *in vitro* amplification of DNA at the logarithmic scale. Various components of the PCR reaction such as Taq DNA polymerase, assay buffer, deoxynucleoside triphosphates, stabilizing agents, and primers make it possible for the DNA template to be amplified sufficiently *in vitro* to attain detectable quantities. PCR can be used for various purposes such as the amplification of human specific DNA sequences, differentiation of species, sub-species and strains, DNA sequencing, detection of mutations, monitoring cancer therapy, detection of bacterial and viral infections, pre-determination of sex, linkage analysis using single sperm cells, ascertaining recombinant clones and studying molecular evolution. PCR is a sensitive technique and therefore highly susceptible to contamination which may result in false positivity. To make PCR a specific, efficient

and cost effective tool for researchers and clinicians the most important component of the PCR is the oligonucleotide primers. Literature searches indicate that insufficient experimental work has been done in the field of bioinformatics especially in the field of nucleic acid sequence analyses. Inadequate experimental data is available (at least in the public domain) for the establishment of primer design strategies. In this review the authors aim to establish various aspects and types of PCR and primer design theory, supported by computational and experimental data.

## PCR Primer Design

Selective amplification of nucleic acid molecules, that are initially present in minute quantities, provides a powerful tool for analyzing nucleic acids (Saiki *et al.*, 1985; Mullis *et al.*, 1987). The polymerase chain reaction is an enzymatic reaction, which follows relatively simple, predictable and well understood mathematical principles. However the scientist often relies on intuition to optimise the reaction. To make PCR an efficient and cost effective tool, some components of PCR such as Taq DNA polymerase, assay buffer, deoxynucleoside triphosphates (dNTPs), stabilizing agents (Sarkar *et al.*, 1990), 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.*, 1993).

DNA template quality or purity is not particularly significant for amplification. Provided DNA does not contain any inhibitor of Taq DNA polymerase, it can be isolated by almost any method (Murray and Thompson, 1980; Sambrook *et al.*, 1989; Kaneko *et al.*, 1989; Mercier *et al.*, 1990; Kawasaki 1990a; Green *et al.*, 1991; Keller *et al.*, 1993; Klebe *et al.*, 1996; Singh and Naik, 2000).

Taq DNA polymerase also plays an important role (Drummond and Gelfand, 1989). Taq DNA polymerase from different suppliers may behave differently because of the different formulations, assay conditions and/or unit definitions. Recommended concentration ranges between 1-2.5 Units/50-100  $\mu$ l reaction (Lawyer *et al.*, 1989) when other parameters are optimal.

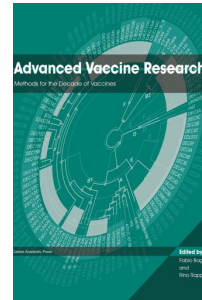
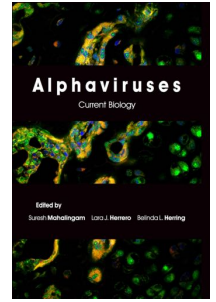
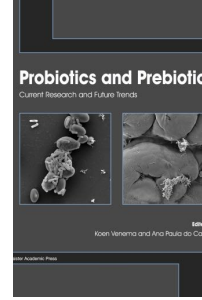
Most of the reviews on PCR optimization (Erlich *et al.*, 1991; Dieffenbach 1993; Roux 1995) consider different parameters of PCR but generally do not discuss basic concepts of PCR primer design. Because of the

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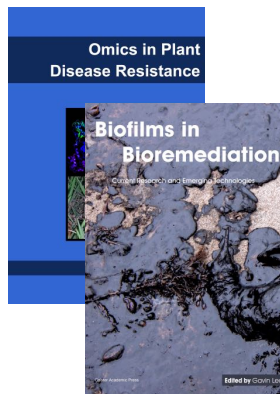
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requirements for different strategies of PCR, more effective PCR studies would be attainable by considering the basic concepts of PCR primer design.

### Primer Length: a Hard Core Factor

Length of a primer is a critical parameter (Wu *et al.*, 1991). The rule-of-thumb is to use a primer with a minimal length that ensures a denaturation temperature of 55-56°C. This greatly enhances specificity and efficiency. For general studies, primers of typically 17-34 nucleotides in length are the best. Primer >16 nucleotides in length are not generally annealed specifically to non-target DNA sequence (e.g. human DNA in an assay for bacterial infection). For example, a short primer sequence, such as a 12 bp oligonucleotide, binds to 200 specific annealing sites in the human genome. (The genome consists of  $3 \times 10^9$  nucleotides:  $3 \times 10^9 / 4^{12} = 200$ ). In contrast, a 20 mer sequence is expected to randomly exist only once every 420 nucleotides and as such, has only a 1 in 400 probability of existing by chance in the human genome. Primers, 18-24 mer are accepted as best in being sequence specific if the annealing temperature of the PCR reactions is set within 5°C of the primer  $T_d$  (dissociation temperature of the primer/template duplex) (Dieffenbach, 1993). Primers work exceptionally well for the sequence with least intra-strand secondary structure. This is because secondary structure impedes primer annealing and extension. Longer primers (28-35 mer) are required only to discriminate homologous genes within different species or when a perfect complementary sequence to all the template is not expected. They could also be used when extra sequence information e.g. a motif binding site, restriction endonuclease site or GC clamp is attached to 5' end. Such extensions do not generally alter annealing to the sequence specific portion of the primer (Sheffield *et al.*, 1989).

Although the following formula is generally used for determining melting temperature ( $T_m$ ):

$$T_m = 4(G+C) + 2(A+T)$$

Frier *et al.* (1986) showed that the nearest-neighbor calculation is better for calculating the melting temperature of longer primers because this also takes account of thermodynamic parameters. Using improved nearest-neighbor thermodynamic values given by John SantaLucia (1995), a good estimate of melting temperature can be obtained for oligonucleotide analysis.

### Terminal Nucleotides Make a Difference

Both the terminals of the primer are of vital importance for a successful amplification. The 3'-end position in the primer affects mispriming. However, for certain reactions, such as amplification refractory mutation system (ARMS), this mispriming is required (Newton *et al.*, 1989; Old, 1991; Tan *et al.*, 1994). Runs (3 or more) of C's or G's at the 3' end of the primer should be avoided as G + C rich sequence leads to mispriming. Complementarity at the 3' end of the primer elevates mispriming as this promotes the formation of a primer dimer artifact and reduces the yield of the desired product (Huang *et al.*, 1992). The stability of the

primer is determined by its false priming efficiency; ideally it should have a stable 5' end and an unstable 3' end. If the primer has a stable 3' end, it will bind to a site which is complementary to the sequence rather than the target site and may lead to secondary bands. It is adequate to have G or C in last 3 bases at 5' termini for the efficient binding of the primer to the target site. This GC clamp reduces spurious secondary bands (Sheffield *et al.*, 1989).

### GC Content, $T_m$ and $T_a$ are Interrelated

GC content, melting temperature and annealing temperature are strictly dependent on one another (Rychlik *et al.*, 1990). GC% is an important characteristic of DNA and provides information about the strength of annealing. A GC of 50-60% is recommended. The value recommended by Dieffenbach (1993) is 45-55%.

### Secondary Structure

An important factor to consider when designing a primer is the presence of secondary structures. This greatly reduces the number of primer molecules available for bonding in the reaction. The presence of hairpin loops reduces the efficiency by limiting the ability to bind to the target site (Singh *et al.*, 2000). It is well established that under a given set of conditions, the relative stability of a DNA duplex structure depends on its nucleotide sequence (Cantor and Schimmel, 1980). More specifically, the stability of a DNA duplex appears to depend primarily on the identity of the nearest-neighbor nucleotides. The overall stability and the melting behavior of any DNA duplex structure can be predicted from its primary sequence if the relative stability ( $\Delta G^0$ ) and the temperature dependent behavior ( $\Delta H^0$ ,  $\Delta C_p^0$ ) of each DNA's nearest-neighbor interaction is known (Marky and Breslauer, 1982). Tinoco *et al.*, (1971, 1973) and Uhlenbeck *et al.*, (1973) have predicted stability and melting behavior of RNA molecules for which they and others have determined the appropriate thermodynamic data. But, to the best of our knowledge, no experimental data is available to support the prediction of the thermodynamic properties of hairpin structures, an important factor to consider when designing a primer. Single stranded nucleic acid sequences may have secondary structures due to the presence of complementary sequences within the primer length e.g. hairpin loops and primer-dimer structures. We have recently shown experimentally that hairpin loops, if present, can greatly reduce the efficiency of the reaction by limiting primer availability and the ability to bind to the target site (Singh *et al.*, 2000). The effect of primer-template mismatches on the PCR has been studied earlier in a Human Immunodeficiency Virus (HIV) model (Kwok *et al.*, 1990). Studies have also been performed for the characterization of hairpins (Marky *et al.*, 1983, 1985), cruciforms (Marky *et al.*, 1985), bulge and interior loops (Patel *et al.*, 1982, 1983).

### Dimers and False Priming Cause Misleading Results

Annealing between the 3' end of one primer molecule and the 5' end of another primer molecule and subsequent

extension results in a sharp background product known as primer dimer. Its subsequent amplification product can compete with the amplification of the larger target. If the primer binds anywhere else than the target site, the amplification specificity is reduced significantly (Breslauer *et al.*, 1986). This leads to a weak output or a smear. This occurs again when some bases at 3' end of the primer bind to target sequence and achieve favorable chances of extension (Chou *et al.*, 1992).

To minimize the possibility of dimers and false priming, PCR is generally performed at high temperature (>50°C), but primers may be extended non-specifically prior to thermal cycling if the sample is completely mixed at room temperature (RT) (Hung *et al.*, 1990). To prevent this occurring the Hot Start® protocol is recommended (Erich *et al.*, 1991). All reagents except one (usually the Taq DNA Polymerase) are mixed at RT. The sample is denatured completely for 3 to 7 min, kept on ice for 2 min and then Taq DNA polymerase is added to start the reaction.

### Know Your Product Before Amplification

PCR product length is directly proportional to inefficiency of amplification (Wu *et al.*, 1991). Primers should be designed so that only small regions of DNA (150-1000 bp) can be amplified from fixed tissue samples or purified plasmid or genomic DNA. The product is ideal for probe hybridization studies (Schowalter and Sommer, 1989). For reverse transcriptase polymerase chain reaction (RT-PCR) as described by Kawasaki (1990b), primers should only be designed in exons taking care that both primers should be on different exons of mRNA to avoid spurious product amplified from contaminating DNA in the mRNA preparation, if any. If the desired restriction enzyme site is not available within the amplified product, it may be incorporated within the primer (Ponce and Michal, 1989; Jung *et al.*, 1990).

### Mismatch to Improve Sensitivity and Specificity

There is a good and a bad aspect to mismatches in primers. Single mismatches at or near the terminal 3' nucleotide of a primer are known to affect both oligonucleotide stability and efficiency of polymerase reaction; mismatches in the primer at or near the 3' terminal end affect PCR more dramatically than mismatches at other positions (Petruska *et al.*, 1988). Generally, mismatches at the 3' end terminal nucleotide reduce or inhibit efficiency of amplification (Kwok *et al.*, 1990; Liu *et al.*, 1994) but studies have shown that a mismatch 3-4 bases upstream of the 3' end of a primer used for the ARMS study actually increases specificity. A mismatch may therefore be deliberately created while designing a primer for ARMS PCR (Old, 1991).

### Nested PCR

Nested PCR is often successful in reducing unwanted products while dramatically increasing sensitivity (Albert and Fenyo, 1990). It is used when the actual quantity of target DNA is very low or when the target DNA is impure. Nested PCR reduces background amplification thereby enhancing target detection. The technique is especially

helpful for amplification of low copy number targets (<100 molecules) and while doing quantitative PCR (Haff, 1994). The process involves one PCR reaction followed by the next PCR extension which amplifies the first PCR product. Two sets of primers are designed. PCR is first carried out using outer primers and subsequently with inner primers positioned within the product obtained in the first extension. It is also possible to perform a nested PCR reaction in a single sample without dilution between the two PCR reactions (Erich *et al.*, 1991). When designing primers for nested PCR, care must be taken to eliminate potential primer dimers and cross dimers within and between inner and outer primer sets.

### Multiplex PCR

This technique involves co-amplification of two or more target sequences within a single sample (Chamberlain *et al.*, 1991; Edwards and Gibbs, 1994). A unique pair of primers for each target is preferred but primers can be designed so that a single primer can amplify different regions with two or more counterparts (Varawalla *et al.*, 1991a; 1991b). When designing primers for multiplex PCR systems, the basic rule is to have similar annealing temperatures and similar GC% of the primers (Nicodeme and Steyaret, 1997). Product length should also be taken into consideration when designing primers so that they can be effectively separated and studied by electrophoresis. Multiplex PCR may be used for detection of genetic disorders (Old *et al.*, 1990; Shuber *et al.*, 1991). Zhu and Clark (1996) demonstrated that addition of competitive primers may dramatically increase PCR amplification efficiency.

### Universal Primers

Molecular biologists are well aware of the exponential increase in the DNA sequence databanks with several thousands bases added every day. Many genes of varied importance have been sequenced in several species. However, the scientific community may require information on such genes in other species, which are used as experimental models. Researchers are often forced to re-sequence genes for new species in order to conduct expression level or other PCR related studies of the gene (Kain *et al.*, 1991) Bulat *et al.* (1992) demonstrated the application of universal primers. Universal primers facilitated the rapid study of novel genes in new models. Rose *et al.*, (1998) demonstrated a new primer design strategy for PCR amplification of unknown targets that are related to multiple-aligned protein sequences. Universal primers are designed in the conserved region of the sequences (Singh *et al.*, 2000). Universal primers should be designed from amino-acid sequences in the regions of lowest degeneracy using a multiple sequence alignment (Nomenclature Committee of the International Union of Biochemistry, 1985). Universal primers were used for differential display of eukaryotic mRNAs by PCR (Liang and Pardee, 1992). A universal primer set for detection of parasitic genomes was also designed using *Dirofilaria immitis* as a test sample (Nagano *et al.*, 1996), whereas Venta *et al.* (1996) designed gene-specific universal

primers for the canine genome. These were used for developing a genetic map of dog-based markers. Universal primers may be used for amplification as well as sequencing in one reaction (Berg and Olaisen, 1994)

### Degenerate Primers

Degeneracy in primer sequence should also be taken into consideration. In fact researchers pursuing the cloning of novel genes often face the problem that only a partial protein sequence is known (Bindon *et al.*, 1998). In these circumstances several procedures can be used, some involve universal primers or reverse translation of the protein sequence into a DNA sequence and the design of primers from this sequence. However, due to redundancy in the genetic code, primer design must account for the ambiguous DNA bases and has to be designed in the region of lowest degeneracy (Kwok *et al.*, 1994). Le Guyader *et al.* (1996) evaluated the effect of degenerate primers in the detection of caliciviruses. Mack and Sninsky (1988) demonstrated the selection of conserved regions encoded by amino acids with minimal codon degeneracy in order to reduce mismatch. 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), homologous genes from different species (Kopin *et al.*, 1990) and related viruses (Mack and Sninsky, 1988; Manos *et al.*, 1989; Shih *et al.*, 1989). A computer program was also developed specifically for degenerate primer design (Chen and Zhu, 1997).

### Software in Primer Design

Most molecular biological applications are aided by software. 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. 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 etc. 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 al.*, 1991; Makarova *et al.*, 1992; Osborne, 1992; Plasterer, 1997; Sze *et al.*, 1998).

### Conclusion

Biological science, and in particular biotechnology, is rapidly changing and cannot achieve its objectives without the help of computer technology and information technology tools. PCR primer design concepts are not new. However constant upgrading and updating of the strategies and methods are essential to maintain rapid and efficient progress. Computational strategies in biotechnology are of particular importance. The algorithms relevant to the efficient design of primers should be modified taking into account experimental data.

### References

- Albert, J., and Fenyo, E.M. 1990. Simple, sensitive and specific detection of human immunodeficiency virus type 1 in clinical specimens by polymerase chain reactions with nested primers. *J. Clin. Microbiol.* 28: 1560-1564.
- Berg, E.S. and Olaisen, B. 1994. Hybrid PCR sequencing: sequencing of PCR products using a universal primer. *Biotechniques* 17: 896-901.
- Bindon, C., Martindale, J., and Mitchell, C. 1998. Biologically generated primer for PCR: PCR primer for unknown sequence. *Nucleic Acid Res.* 26(13): 3305-3358.
- Breslauer, K.J., Ronald, F., Blocker, H., and Marky, L.A. 1986. Predicting DNA duplex stability from the base sequence. *Proc. Natl. Acad. Sci.* 83: 3746-3750.
- Bulat, S.A., Kobaev, O.K., Mironenko, N.V., Ibatullin, F.M., Luchkina, L.A., and Suslov, A.V. 1992. Polymerase chain reaction with universal primers for studying genomes. *Genetika* 28: 19-28.
- Chamberlain, J.S., Gibbs, R.A., Ranier, J.E., and Caskey, C.T. 1991. Detection of gene deletions using multiplex polymerase chain reactions. *Meth. Molec. Biol.* 9: 299-312.
- Chang, J.G., Lu, J.M., Huang, J., Chen, J.T., Liu, H.J., and Chang, C.P. 1995. Rapid diagnosis of  $\beta$ -thalassaemia by mutagenically separated polymerase chain reaction (MS-PCR) and its application to prenatal diagnosis. *Br. J. Haemat.* 91: 602-605.
- Chen, H., and Zhu, G. 1997. Computer program for calculating the melting temperature of degenerate oligonucleotides used in PCR or hybridization. *Biotechniques* 21: 134-140.
- Chou, Q., Russell, M., Birch, D.E., Raymond, J., and Bloch, W. 1992. Prevention of pre-PCR mis-priming and primer dimerization improves low-copy number amplifications. *Nucleic Acids Res.* 20: 1717-1723.
- Dieffenbach, C.W., Lowe, T.M.J., and Dveksler, G.S. 1993. General concepts for PCR primer design. In: *PCR Methods and Applications*, Cold Spring Harbor Laboratory 3: S30-S37.
- Drummond, R., and Gelfand, D.H. 1989. Isolation, characterization and expression in *Escherichia coli* of the DNA polymerase gene from *Thermus aquaticus*. *J. Biol. Chem.* 264: 6427-6436.
- Edwards, M.C., and Gibbs, R.A. 1994. Multiplex PCR: Advantages, development and applications. *PCR Methods Applic.* 3: S65-S75.
- Engelke, D.R., Hoener, P.A., and Collins, F.S. 1988. Direct sequencing of enzymatically amplified human genomic DNA. *Proc. Natl. Acad. Sci.* 85: 544-550.
- Erlich, H.A., Gefland, D., and Sninsky, J.J. 1991. Recent advances in the polymerase chain reaction. *Science* 252: 1643-1651.
- Freier, S.M., Kierzek, R., Jaeger, J.A., Sugimoto, N., Caruthers, M.H., Neilson, T., and Turner, D.H. 1986. Improved free-energy parameters for predictions of RNA duplex stability. *Proc. Natl. Acad. Sci., USA.* 83: 9373-9377.
- Green, C.E., Lund, J.K., and Manos, M.M. 1991. PCR amplification from paraffin-embedded tissues: Recommendations on fixatives for long term storage and prospective studies. *PCR Methods Appl.* 1: 46-50.
- Haff La. 1994. Improved quantitative PCR using nested primers. *PCR Methods Appl.* 3: 322-327.
- He, Q., Marjamaki, M., Soini, H., Mertsola, J., and Viljanen, M.K. 1994. Primers are decisive for sensitivity of PCR. *Biotechniques.* 17: 82-87.
- Huang, M., Arnheim, N., and Goodman, M.F. 1992. Extension of base mismatches by Taq DNA polymerase: Implications for single nucleotide discrimination in PCR. *Nucleic Acids Res.* 20: 4567-4573.
- Hung, T., Mak, K. and Fong, K. 1990. A specificity enhancer for PCR. *Nucleic Acids Res.* 18: 4953-4959.
- Jung, V., Pestka, S.B., and Pestka, S. 1990. Efficient cloning of PCR generated DNA containing terminal restriction endonuclease sites. *Nucleic Acid Res.* 18: 6156-6160.
- Kain, K.C., Orlandi, P.A., and Lanar, D.E. 1991. Universal promoter for

- gene expression without cloning Expression-PCR. *BioTechniques* 10: 366-374.
- Kaneko, S., Feinstone, S.M., and Miller, R.H. 1989. Rapid and sensitive method for the detection of serum hepatitis B virus DNA using the polymerase chain reaction technique. *J. Clin. Microbiol.* 27: 1930-1933.
- Kawasaki, E.S. 1990a. Sample preparation from blood, cells and other fluids, In: PCR Protocols, a guide to methods and applications, Innis, M.A., Gelfand, D.H., Sninsky, J.J. and White, T.J. (eds), Academic Press Inc., Berkeley CA, pp. 146-152.
- Kawasaki, E.S. 1990b. Amplification of RNA, In: PCR Protocols, a guide to methods and applications, Innis, M.A., Gelfand, D.H., Sninsky, J.J. and White, T.J. (eds), Academic Press Inc., Berkeley CA, pp. 21-27.
- Keller, G.H., Cumming, C.U., Huang, D.P., Manak, M.M., Ting, R. 1988. A chemical method for introducing Haptens onto DNA probes. *Anal. Biochem.* 170: 441-450.
- Klebe, R.J., Grant G.M., Grant, A.M., Garcia, M.A., Giambernardi, T.A., and Taylor, G.P. 1996. RT-PCR without RNA isolation. *Biotechniques* 21: 1094-1100.
- Kumar A., and Mishra B.N. 1997. Primer Premier 4. Biotech Software and Internet Journal 3: 31-38.
- Kwok S., Kellogg D.E., McKinney, E., Spasic, D., Levenson, C., Sninsky, J.J. 1990. Effects of Primer-template mismatches on the polymerase chain reaction: Human Immunodeficiency virus type I model studies, *Nucleic Acids Res.* 18: 999-1005.
- Kwok, S., Chang, S.Y., Sninsky, J.J., and Wong, A. 1994. A guide to the design and use of Mismatched and Degenerate primers. *PCR Methods and Appl.* S539-S547.
- Lawyer, F.C., Stoffel, S., Saiki, R.K., Myambo, K., Drummond, R., and Gelfand, D.H. 1989. Isolation, characterization, and expression in *Escherichia coli* of the DNA polymerase gene from *Thermus aquaticus*. *J. Biol. Chem.* 15: 6427-6437.
- Le Guyader, F., Estes, M.K., Hardy, E., Neill, F.H., Green, J., Brown, D.W., and Atmar, R.L. 1996. Evaluation of a degenerate primer for the PCR detection of human caliciviruses. *Arch. Virol.* 141: 2225-2235.
- Liang, P., and Pardee, A. 1992. Differential display of eukaryotic mRNAs by PCR. *Science* 257: 967-971.
- Linz, U., Delling, U., and Rubsamem-Waigmann 1990. Systematic studies on parameters influencing the performance of the polymerase chain reaction. *J. Clin. Chem. Clin. Biochem.* 28: 5-13.
- Liu, Q., Thorland E.C., and Sommer, S.S. 1997. Inhibition of PCR amplification by a point mutation down stream of a primer. *Biotechniques* 22: 292-298.
- Lowe, T., Shareifkin, J., Yang, S.Q., and Dieffenbach, C.W. 1990. A computer program for selection of oligonucleotide primers for polymerase chain reaction. *Nucleic Acids Res.* 18: 1757-1761.
- Lucas, K.M., Busch, S.M., and Thompson, J.A. 1991. An improved microcomputer program for finding gene or gene family-specific oligonucleotides suitable as primers for polymerase chain reactions or as probes. *CABIOS* 7: 525-529.
- Mack, D.H., and Sninsky, J.J. 1988. A sensitive method for the identification of uncharacterized viruses related to known virus groups: Hepadnavirus model system. *Proc. Natl. Acad. Sci.* 85: 6977-6981.
- Makarova, K.S., Mazin, A.V., Wolf, Y.I., and Soloviev, V.V. 1992. DIROM: An experimental design interactive system for directed mutagenesis and nucleic acid engineering. *CABIOS* 8: 425-431.
- Manos, M.M., Ting, Y., Wright, D.K., Lewis, A.J., Broker, T.R., and Wolinsky, S.M. 1989. Use of polymerase chain reaction amplification for the detection of genital human papillomaviruses. *Cancer Cells (Molecular diagnostics of Human Cancer)* 7: 209-214.
- Marky, L.A., Blumentfeld, K.S., Kozlowski, S., and Breslauer, K.J. 1983. Salt-dependent conformational transitions in the self-complementary deoxydodecanucleotide (CGCAATTCGCG): evidence for hairpin formation. *Biopolymers* 22: 1247-57.
- Marky, L.A., Kallenbach, N.R., Seeman, N.C., Haasnoot, C.A.G., and Breslauer, K.J., 1985. Fourth Conversation in Biomolecular stereodynamics, Ed. Sharma R.H. Adenine Guilderland, NY.
- Mercier, B., Gaucher, C., Feugeeas, O., Mazurier, C. 1990. Direct PCR from whole blood without DNA extraction. *Nucleic Acid Res.* 18(19): 5908-5912.
- Moore, R.E., Shepherd, J.W., and Hoskins, J. 1990. Design of PCR primers that detect only mRNA in the presence of DNA, *Nucleic Acids Res.* 18: 1921-1926.
- Mullis, K.B., and Faloona, F. 1987. Specific synthesis of DNA *in vitro* via a polymerase-catalyzed chain reaction. *Methods Enzymol.* 155: 335-351.
- Murray, M., and Thompson, W. 1980. Rapid isolation of high molecular weight plant DNA, *Nucleic Acids Res.* 8: 4321-4325.
- Nagano, I., Zhiling, W., Nakayama, M., Takahashi, Y. 1996. A simple method to design PCR primer to detect genomic DNA of parasites and its application to *Diofilaria immitis*. *Mol. Cell. Probes.* 10: 423-425.
- Nelson, J.W., Martin, F.H., and Tinoco, I., Jr. 1981. DNA and RNA oligomer thermodynamics: the effect of mismatched bases on double-helix stability. *Biopolymers.* 20: 2509-2531.
- Newton, C.R., Graham, A., Heptinstall, L.E., Powell, S.J., Summers, C., Kalsheker, N., Smith, J.C., and Markham, A.F. 1989. Analysis of any point mutation in DNA: The amplification refractory mutation system (ARMS). *Nucleic Acid Res.* 17: 2503-2516.
- Nicodeme, P. and Steyaert J.M. 1997. Selecting optimal oligonucleotide primers for multiplex PCR *ISMB* 5:210-213.
- Nomenclature Committee of the International Union of Biochemistry 1985. Nomenclature for incompletely specified bases in nucleic acid sequences. *Eur. J. Biochem.* 150: 1-5.
- O'Hara, P.J., and Venezia, D. 1991. PRIMGEN: a tool for designing primers from multiple alignments. *CABIOS* 7: 533-534.
- Old, J.M., 1991. Detection of Mutations by the Amplification Refractory Mutation System (ARMS), *Meth. Mol. Biol.* 9: 77-84.
- Osborne, B.I. 1992. Hyper PCR: A Macintosh Hypercard program for determination of optimal PCR annealing temperature. *CABIOS* 8: 33-34.
- Patel, D.J., Kozlowski, S.A., Marky, L.A., Rice, J.A., Broka, C., Itakura, K., and Breslauer, K.J. 1982. Structure and energetics of a hexanucleotide duplex with stacked trinucleotide ends formed by the sequenced (GAATTCGCG). *Biochemistry.* 21: 451-455.
- Patel, D.J., Kozlowski, S.A., Marky, L.A., Rice, J.A., Broka, C., Itakura, K., and Breslauer, K.J. 1983. Topics in Nucleic acid structure, Ed. Neidle, S. Wiley, NY. pp.81-136.
- Petruska, J., Goodman, M.F., Boosalis, M.S., Sowers, L.C., Cheong, C., Tinoco, I.Jr. 1988. Comparison between DNA melting thermodynamics and DNA polymerase fidelity. *Proc. Natl. Acad. Sci.* 85: 6252-6256.
- Plasterer, T.N. 1997. Primerselect: Primer and probe design. *Methods Mol. Biol.* 70: 520-527.
- Ponce, M.R., and Micol, J.L. 1992. PCR amplification of long DNA fragments, *Nucleic Acids Res.* 20: 623-627.
- Rose, T.M., Schultz, E.R., Henikoff, J.G., Peitrokovski, S., McCallum, C.M., and Henikoff, S. 1998. Consensus degenerate hybrid oligonucleotide primers for amplification of distantly related sequences. *Nucleic Acid Res.* 26: 1628-1635.
- Roux, K.H. 1995. Optimization and troubleshooting in PCR in: PCR methods and applications. Cold spring Harbor laboratory. 5185-5194.
- Rychlik, W., and Rhoads, R.E. 1989. A computer program for choosing optimal oligonucleotides for filter hybridization, sequencing and *in vitro* amplification of DNA. *Nucleic Acids Res.* 17: 8543-8551.
- Rychlik, W., Spencer, W.J., and Rhoads, R.E. 1990. Optimisation of annealing temperature for DNA amplification *in vitro*. *Nucleic Acids Res.* 18: 6409-6412.
- Saiki, R.K., Scharf, S., Faloona, F., Mullis, K.B., Horn, G.T., Erlich, H.A., and Arnheim, N. 1985. Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* 230: 1350-1354.
- Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989. *Molecular Cloning, A laboratory manual*, 2<sup>nd</sup> Ed., Cold Spring Harbor Laboratory Press.
- SantaLucia, J.Jr., Allawi, H.T. and Seneviratne, P.A. 1996. Improved Nearest-Neighbor parameters for predicting DNA duplex stability. *Biochemistry* 35: 3555-3562.
- Sarkar, G., Kapelner, S., and Sommer, S.S. 1990. Formamide can dramatically improve the specificity of PCR. *Nucleic Acids Res.* 18: 7465-7468.
- Schwalter, D.B., and Sommer, S.S. 1989. The generation of radiolabelled DNA and RNA probes with polymerase chain reaction, *Anal. Biochem.* 177: 90-94.
- Sheffield, V.C., Cox, D.R., Lerman, L.S., and Myers, R.M. 1989. Attachment of a 40 base pair G+C rich sequence (GC-clamp) to genomic DNA fragments by polymerase chain reaction results in improved detection of single base changes. *Proc. Natl. Acad. Sci.* 86: 232-236.
- Shuber, A., Skoletsky, J., Handelin, B. 1991. A rapid method for simultaneous analysis of multiple point mutations within the Cystic Fibrosis gene. *Am. J. Hum. Genet.* 49: 189-194.
- Singh, V.K., Mangalam, A.K., Dwivedi, S., and Naik, S. 1998. Primer Premier: Program for design of degenerate primers from a protein sequence, *Biotechniques* 24: 318-319.
- Singh V.K., and Naik, S. 2000. Room temperature stable polymerase chain reaction kit for detection of Hepatitis B Virus. *Clin. Chim. Acta.* 301: 225-227.
- Singh, V.K., Govindarajan, R., Naik, S., and Kumar, A. 2000. The effect of hairpin structure on PCR Amplification efficiency. *Mol. Biol. Today.* 1: 657-659.
- Singh, V.K., Manglam, A.K., Kumar, A., and Naik, S. 2001. Universal primers can amplify tumour necrosis factor gene across species. *Mol. Biol. Today* 2: 11-12.
- Singh V.K., and Naik, S. 2001. Simultaneous amplification of DNA and RNA virus using Multiplex PCR system. *Clin. Chim. Acta.* (In Press).
- Sze, S.H., Raytberg, M.A., Gelfand, M.S., Mironov, A.A., Astakhova, T.V.,

- Pevzner, P.A. 1998. Algorithms and software for support of gene identification experiments. *Bioinformatics* 14: 14-19.
- Tamura, T., Holbrook, S.R., and Kim, S.H. 1991. A Macintosh computer program for designing DNA sequences that for specific peptides and proteins. *BioTechniques*. 10: 782-784.
- Tan, J.A.M.A., Tay, J.S.H., Lin, L.I., Kham, S.K.Y., Chia, J.N., Chin, T.M., Norkamov, B.T., Aziz, A.O.B., and Wong, H.B. 1994. The amplification refractory mutation system (ARMS): a rapid and direct prenatal diagnostic technique for  $\beta$ -thalassaemia in Singapore. *Prenat. Diagn.* 14: 1077.
- Tinoco, I.Jr., Borer, P.N., Dengler, B., Levin, M.D., Uhlenbeck, O.C., Crothers, D.M., and Bralla, J. 1973. Improved estimation of secondary structure in ribonucleic acids. *Nat. New Biol.* 14: 40-41.
- Tinoco, I.Jr., Uhlenbeck, O.C., and Levine, M.D. 1971. Estimation of secondary structure in ribonucleic acids. *Nature* 9: 362-367.
- Uhlenbeck, O.C., Borer, P.N., Dengler, B., and Tinoco, I.Jr. 1973. Stability of RNA hairpin loops: A 6-C m-U 6. *J. Mol. Biol.* 5: 483-496.
- Varwall, N.Y., Old, J.M., and Weatherall, D.J. 1991b. Rare  $\beta$ -thalassaemia mutations in Asian Indians, *Br. J. Haemat.* 79: 640-644.
- Varwalla, N.Y., Old, J.M., Sarkar, R., Venkatesan, R., and Weatherall, D.J. 1991a. The spectrum of  $\beta$ -thalassaemia mutations on the Indian subcontinent: the basis for prenatal diagnosis, *Br. J. Haemat.* 78: 242-247.
- Venta, P.I., Brouillette, J.A., Yusbasiyan-Gurkan, V., and Brewer, G.J. 1996. Gene specific universal mammalian sequence – tagged sites: application to the canine genome. *Biochem. Genet.* 34: 321-341.
- Wilks, F.F., Kurban, R.R., Hovens, C.M., and Ralph, S.J. 1989. The application of the polymerase chain reaction to cloning members of the protein tyrosine kinase family. *Gene.* 85: 67-74.
- Wilson, J.T., Wilson, L.B., deRiel, J.K., Villa-Komaroff, L., Efstratiadis, A., Forget, B.G., and Weissman, S.M. 1978. Insertion of synthetic copies of human globin genes into bacterial plasmids. *Nucleic Acids Res.* 5: 563-581.
- Wrischnick, L.A., Higuchi, R.G., Stoneking, M., Erlich, H.A., Arnheim, N., and Wilson, A.C. 1987. Length mutations in human mitochondrial DNA: Direct sequencing of enzymatically amplified DNA. *Nucleic Acids Res.* 15: 529-542.
- Wu, D.Y.W., Ugozzoli, B.K., Pal, J., Qian, and Wallace, R.B. 1991. The effect of temperature and oligonucleotide primer length on specificity and efficiency of amplification by the polymerase chain reaction. *DNA Cell Biol.* 10: 233-238.
- Zhu, K.Y., and Clark, J.M. 1996. Addition of competitive primer can dramatically improve the specificity of PCR amplification of specific alleles. *Biotechniques.* 21: 586-590.