Molecular Biology *Today* (2000) 1(3): 67-69.

The Effect of Hairpin Structure on PCR Amplification Efficiency

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Abstract

Design of primers for efficient DNA amplification using polymerase chain reaction is critical. Therefore, evaluation of hairpin structures while designing position dependent primers needs special attention. Here, we present the data on the effect of hairpins on DNA amplification using polymerase chain reaction. X-antigen of Hepatitis B Virus has been used as a model. Studies showed that hairpin loop with less than three complementary nucleotides did not have any effect whereas primer length is directly proportional to the hairpin size. Most important characteristic of hairpin structure affecting the amplification is loop size. There should be no complementarity among the primers at their 3' end because this greatly increases the possibility of spurious products by amplifying themselves, thereby, decreasing the amplification efficiency. Hairpin structures greatly affect the primer designing theory having direct relation to amplification.

Introduction

The polymerase chain reaction (PCR) (Saiki *et al*., 1985) has proven to be sensitive and specific assay for the detection of minute quantity of nucleotide sequences. Efficacy and sensitivity of PCR largely depend on the efficiency of the primers. The primers which are unique for the target sequence to be amplified should fulfill certain criteria like primer length, GC %, annealing and melting temperature, 5' end stability, 3' end specificity (Dieffenbach *et al*., 1993). Another important factor to be considered while designing a primer is secondary structure. Single stranded nucleic acid sequences may have secondary structure (hairpin loops and primer dimer) due to the presence of complementary sequences within its length. Hairpin loops, if present can greatly reduce the efficiency of the reaction by limiting its availability and ability to bind to the target site. Taq DNA polymerase may extend the 3' end of the primer using the 5' end as the template with hairpin loops having loop structure at 3' end of the primer with the 5' end protruding as a single strand. This leads to stabilization of the hairpins, exhaustion of reactants in the mixture and appearance of non-specific products in the reaction. Effect of primer-template mismatching on the PCR has been studied earlier in Human Immunodeficiency Virus (HIV) model (Kwok *et al*., 1990). In the present paper, we have investigated the effect of hairpins on DNA amplification of X-gene of Hepatitis B Virus (HBV) using PCR. A correlation has been generated with reduction in amplification efficiency and characteristics of hairpin loops.

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Results

Amplification efficiency was determined by measuring the PCR products from the second round of amplification. A strong band was seen in lanes 4, 6, 8, 11 as compared to weak bands in lanes 3, 7, 9 and 10. Two distinct bands could be observed in lane 8. This could be due to false priming developed due to modification in the primer sequence. Bands 11, 13, 14 corresponding to primer number 11, 14, 15 showed that PCR efficiency was not affected in the presence of a hairpin structure at 3' end of the primer. Hairpin loop of less than three nucleotides complementarity does not effect the quantity of the product (Figure 1). Primer with hairpins of four or more nucleotides stem length exhibited severely reduced amplification efficiency (40-90%) compared with the control primers (Figure 2). In the present study, 3' end hairpin loop primers are also studied. A primer with stem length three designed with internal as well as external loops showed same amount of amplification. The study indicated that in designing of

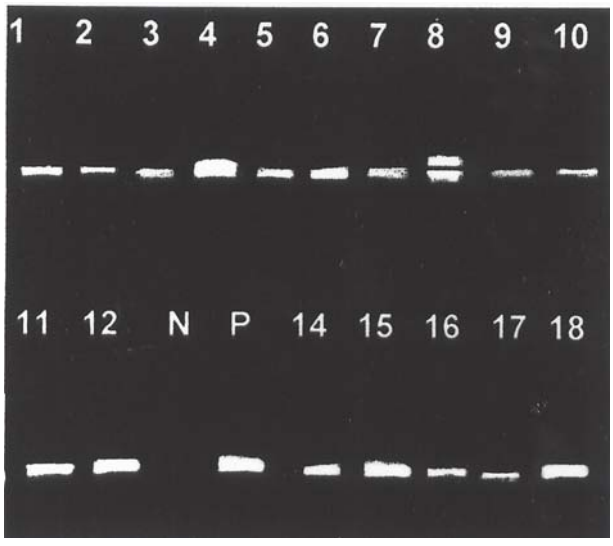

Figure 1. Representative agarose electrophoretic gel showing PCR amplification using modified primers. Lane 1-12, PCR products using primer 1-12 with reverse primer 13; Lane 13-18, PCR products using primer 14-18 with reverse primer 19; Lane N, negative control and Lane P, Positive control.

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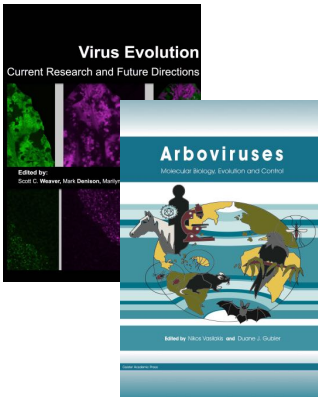

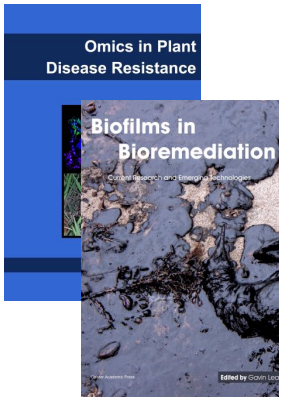

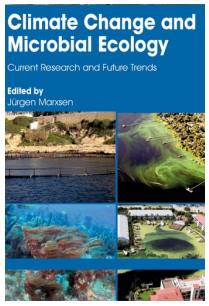

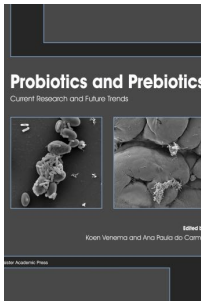

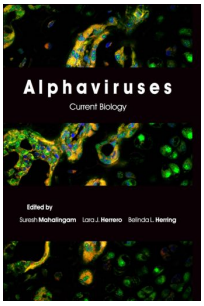

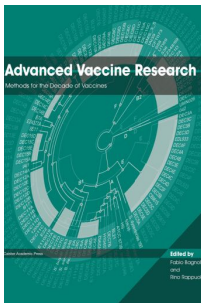

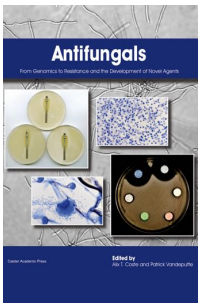

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Table 1. Oligonucleotide Primer Sequences Used in the Study

Primer No.	*Stem length*	*Loop size*	*Bonding strength (%)*	*ΔG*	*Primer sequence*
1st Set of Primers					
Primer 1	4	4	50	-1.9	5' - GTT TTG CTC GCC AAC GGT TTG G –3'
Primer 2	4	8	50	-2.3	5' – GTT TTG CCA ACA CCC GGT TTG G –3'
Primer 3	4	12	50	-1.4	5' – GCC TTG CTC GCA CCC GGT AAG G –3'
Primer 4	4	4	75	-3.3	5' – GTT TTG CTC GCC GAC GGT TCG G –3'
Primer 5	4	4	100	-4.7	5' – GTT TTA ATC GCC CCC GGT GGG G –3'
Primer 6	5	4	60	-3.4	5' - GTT TTG CTC GAG ACC GAT CTC G –3'
Primer 7	5	8	60	-3.8	5' – GTT TCG AGA GCA CCC GGT CTC G –3'
Primer 8	5	12	60	-2.8	5'– TTC GTG GCT CGC ACC CGG TCA CGA -3'
Primer 9	5	8	80	-5.8	5'- GTT TCG AGC GCA CCC GGG CTC G –3'
Primer 10	5	8	20	-1.4	5'- GTT TCT ATA GCA CCC GGT ATA G –3'
Primer 11	3	12	66	0.8	5'- GTT CCA CTC GCA CCC GGT CTG G –3'
Primer 12 Sense Control)	0	0	0		5'- GTT TTG CTC GCA CCC GGT CTG G –3'
Primer 13 (anti-sense Control)	0	0	0		5'- GAG GAA GTG GAC ACG GGT TAG –3'
2nd Set of Primers					
Primer 14	3	5	66.6	1.5	5'- TTT ACT CGC AGC CGG TCT G – 3'
Primer 15	3 (internal)	3	66.6	2.1	5'- TTT GCT CGC AGC CGG TCT G – 3'
Primer 16	4	3	50	0.5	5'- TTT GAT CGC AGA CGG TCT G – 3'
Primer 17	5	3	80	-4.5	5'- TTT GAT CGC AGA CGC TGC G – 3'
Primer 18 (sense Control)	0	0	0		5'- TTT GCT CGC AGC CGG TCT G – 3'
Primer 19 (anti-sense Control)	0	0	0		5' – ATT AGG CAG AGG TGA AAA AG – 3'

PCR primers, hairpins of four or more nucleotides stem length should be avoided.

Discussion

Step-wise multiple regression analysis comparing amplification efficiency with hairpin stem length, loop size, GC% and ΔG, showed that stem length was the most important characteristic ($p<0.05$, $r = 0.58$). This was also confirmed by full model regression analysis taking amplification as constant and other characteristics of the primers to be variable. It shows significance of stem length ($r = 0.74$) over the other characteristics ($r = 0.63$). ΔG was not found significant in this test ($r = 0.43$). So, there was not much effect on amplification by changing ΔG of the primers. However, Bresauler et al. (1986), Groebe and Uhlenbeck (1988) showed effect of ΔG on predicting DNA-DNA duplex study from the nucleotide sequence and characterization of RNA hairpin loop stability, respectively. These data were not based on the experimental studies. Primers containing hairpins of three nucleotides stem length amplified with statistically insignificant differences as compared to control primers containing no hairpins.

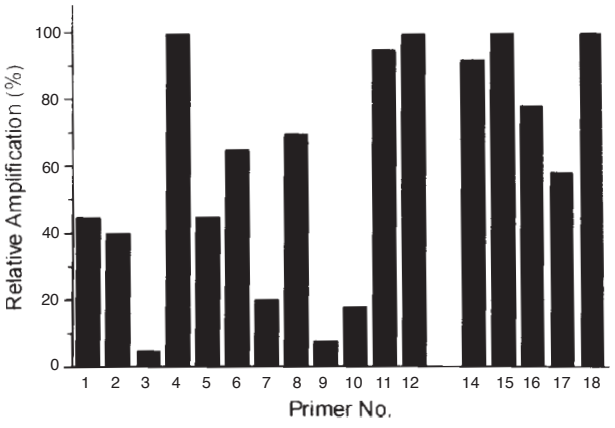

Figure 2. Histogram showing relative amplification of different primers. Sequences of the primers are given in Table 1.

Conclusion

This study confirms the importance of various characteristics viz. loop size, stem length, GC% in a hairpin structure. These parameters play significant role in deciding effect of hairpin structure on PCR amplification efficiency. These parameters can be further correlated in various algorithms used in the software used for primer designing. At present most of the primer designing softwares do not consider these parameters while evaluating potential pimers.

Experimental Procedures

Primer Designing

Oligonucleotide primers were designed using Primer Premier 4.1 (PREMIER Biosoft International, Palo Alto, CA) (Kumar and Mishra, 1997). Two pairs of control primers (Primer 12, 13 and Primer 18,19) were designed with no hairpin structure. The 3' end of the sense primer of this pair was modified by introducing hairpin structures exhibiting a range of hairpin characteristics including varying stem lengths, loop sizes and GC%. The primers were designed with hairpin including a ΔG ranging from –5.4 to 0.8 to test whether hairpin ΔG is a good measure of amplification efficiency. (Table 1).

Target Sequence Generation

Modifications in the primer sequence resulted in loss of homology to the target sequence. To correct this, the template, plasmid pAM6 containing the HBV genome (ATCC, Rockville, MD, USA) was initially amplified using the primers with secondary structure under less stringent conditions (denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 1 min for 35 cycles) to tolerate the primer/template mismatch. The product was sequenced using the ABI Prism 377 DNA sequencer (Applied Biosystems, USA) for confirmation of the sequence. The product, which contained complete homology to the primers, was used as the template for subsequent amplifications.

PCR Amplifications

Amplification was carried out in 50 μl reaction volumes containing 50 pmoles of each primer, 1 unit of Taq DNA polymerase (Bangalore Genei Pvt. Ltd., India) and 25 μM of each of the four dNTPs in a buffer containing 10 mM Tris pH 8.3, 50 mM KCl, and 2.5 mM $MgCl_2$. The primers were evaluated by 10% polyacrylamide gel electrophoresis for observing their hairpin structure due to change in mobility. Template concentration was determined by spectrophotometry and serially diluted to quantify number of copies used in individual reactions. A uniform thermal profile was used for the amplification of the samples with denaturation at 95°C for 1 min, annealing at 56°C for 1 min, extension at 72°C for 1 min for 30 cycles and a final extension at 72°C for 5 min. All amplifications were performed in duplicate and repeated separately with multiple stock solutions. Amplification products were analyzed by 2.5% NuSieve agarose gel electrophoresis with ethidium bromide staining and quantified by optical density measurement at 260 nm (Hitachi UV spectrophotometer). The product of 545 bp obtained from first round of amplification was also confirmed by sequencing.

Acknowledgement

The authors acknowledge JICA grant in aid to SGPGI, Lucknow for laboratory infrastructural facilities; and facilities of the Department of Biotechnology, Government of India, New Delhi under its M.Sc. Biotechnology program and Distributed Bioinformatics sub-centre at the School of Biotechnology, D.A. University, Indore.

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