OPTIMIZATION OF PCR THROUGH MANIPULATION OF CYCLE TIMES AND INCLUSION OF FORMAMIDE

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ABSTRACT

Polymerase chain reaction (PCR) is an in vitro technique to produce million fold copies of a particular segment of DNA. PCR should optimally yield a unique product unless designed otherwise. Artefacts appear many times, which affect the success of downstream applications. Prevention of non specific amplification using formamide and manipulation of cycling time is discussed in this article.

INTRODUCTION

Polymerase chain reaction (PCR) is an *in vitro* cell free technique to produce a large number of copies of a specific segment of DNA. The technique of PCR (Mullis, 1983) evolved during four decades after the establishment of double stranded helix structure of DNA by Watson and Crick (1953). Kleppe (1971), an associate of Hargobind Khorana probably introduced the concept of PCR; with two primers, and cycles of repair synthesis after addition of DNA polymerase in each round. Earlier PCR relied upon DNA polymerase I enzyme of *Escherichia coli* or rather its 'Klenow fragment', which was thermolabile and needs to be added afresh before each cycle. Chien *et.al*. (1976) purified DNA polymerase from *Thermus aquaticus* (*EC 2.7.7.7),* which later became famous as 'Taq' polymerase. The molecule had a weight in the range of 63-68 KDa with an optimum temperature of activity at 80°C in a buffer of pH of 8.0. Ever since *Taq* polymerase was included in PCR eliminating the need to add DNA polymerase before initiation of each cycle, it gained acceptance and was used for a myriad of downstream applications.

Even though the PCR optimally should yield a unique product (unless designed otherwise) many times unintended artefacts also appear. It becomes important to avoid such spurious amplifications so as to improve the success rate of downstream applications like molecular cloning, PCR-RFLP etc. This article attempts to throw light into the optimisation of PCR, when multiple spurious amplicons appear along with the expected product.

MATERIALS AND METHODS

PCR reactions were set up so as to contain 20-100 ng of template DNA, 5 nM each of forward and reverse primers (Sigma), 200 mM of each dNTP's (Fermentas), 2.5 mM of $MgCl_2$ (Sigma),

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0.75 U Taq polymerase (Sigma), polymerase buffer (Sigma) to a final concentration of 1X, diluted with autoclaved Millipore water in a volume of 10 μL which was amplified in a Bio-Rad thermal cycler.

'Gradient' is an option in most thermal cyclers for optimization of cycling conditions. The positions for reaction tubes are arranged rows and columns. The positions in a row can be programmed to maintain one particular temperature and is called a block. When gradient option is set, the temperature remain constant in a row and varies along a column of tube positions. The programme used for gradient PCR was as follows; initial denaturation at 94°C for three minutes, followed by 35 cycles of denaturation at 94°C for one minute, annealing temperature over the gradient for 30 seconds and extension at 72°C for 1 minute with a final extension time of 2 minutes at 72°C. The gradient used in this experiment is presented in table (1).

Sl No	Block	Temperature (°C)	
		FUT1	HSD 17β
1	A	65.0	59.0
2	B	64.3	58.4
3	C	63.2	57.4
4	D	61.4	55.8
5	E	59.3	53.9
6	F	57.7	52.5
7	G	56.6	51.5
8	H	56.0	

Table 1: Block temperatures in gradient PCR

The loci under study were segments from Fucosyl transferase 1 (FUT1) and 17β Hydroxy steroid dehydrogenase (HSD17β) genes, which are candidate genes (Spotter and Distl., 2006) for litter traits in pigs. The primer pair FUT1 F: 5'-GCCGCCACCTCTGTCTGACC-3' and FUT1 R: 5'-TACCCCCTGGGCCTCTTGCC-3' were designed from Genbank accession no. L50534.1 using Primer-Blast tool (available at http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome). The forward and reverse primers had a length of 20 bases each with a GC content of 70 percent. The melting temperature (T_m) of forward primer was 65.23°C and that of reverse primer was 66.0°C. The T_m of an oligonucleotide is the temperature at which half of the molecules remain free of secondary structures. The expected product size for this amplicon was 578 bp.

A segment of HSD 17β gene was amplified using the primers described by Jacobs *et.al*. (2002). The nucleotide sequence of forward primer was 5'- CTCCCACCCCACCTGTTC-3' and that of the reverse primer was 5' CCGTTCACCACCCCTCCTC-3', with an expected product size of 273 bp. The forward primer was 18 nucleotides long while the reverse primer had a length of 19 nucleotides. The GC content was 66.67percent and 68.42 percent respectively for the forward and reverse primers. The melting temperature (T_m) of forward primer was 59.24°C while that of the reverse primer was 61.96°C.

Gradient PCR was done across a block temperature of 56°C and 65°C for FUT1 locus and 50 - 59°C in the case of HSD17β locus. The amplified products were loaded into 2 percent agarose (SRL labs) gel with pre incorporated ethidium bromide (SRL labs) and electrophoresed in Tris acetate EDTA buffer at 85 volt for 45 minutes before being photographed in Geldoc (Pharmacia). The output of gradient PCR for FUT1

is presented in figure (1) and that of HSD 17β in figure (2). Several non specific products could be observed in both the loci. In order to suppress spurious amplifications, formamide was introduced into the reaction mix, along with the reduction of extension time. The composition of reaction mixture was modified by including 0. 75 percent V/V of formamide (USB) and was used to amplify FUT1 (Fig: 3) and HSD 17 β loci (Fig: 4). However mere addition of formamide did not remove all non specific amplicons and a new PCR programme was used in case of HSD 17 β gene (Table 2).

Sl No	Step	Temperature	Duration	No. of cycles
1	Initial denaturation	94°C	3 minutes	1
2	Denaturation	94°C	45 seconds	3 cycles
3	Annealing		15 seconds	
4	Extension	72°C	30 seconds	
5	Denaturation	94°C	45 seconds	32 cycles
6	Annealing		15 seconds	
7	Extension	72°C	30 seconds	
8	Final extension		2 minutes	1
9	END/ Final hold*		∞	

Table 2: Modified PCR programme used for HSD 17β locus

RESULTS

The result from gradient PCR of FUT1 locus is given in Fig (1) and that of HSD 17 β is provided in Fig (2). Four non specific products lower in molecular mass than the expected amplicon (578 bp) were observed in case of FUT1 locus. A product in excess of 1 Kb also appeared when the annealing temperature was below 59.3°C. The larger product failed to amplify when the annealing time was reduced from 30 seconds to 15 seconds, with the simultaneous reduction of extension time from one minute to 45 seconds. A second gradient PCR was performed with formamide (0.75 percent V/V) incorporated in the reaction mixture using the modified programme. Problem of spurious amplifications could be successfully controlled in FUT1 locus (Fig: 3) and an annealing temperature was selected for subsequent amplification of this locus.

In case of HSD 17 β locus, four non specific products, all above the intended product of 273 bp was observed. Addition of formamide and reduction of extension and annealing times did not quite well remove non specific amplifications (Fig: 5, lanes 5 to 7). Annealing time was reduced to 15 seconds and the extension time was reduced to 30 seconds. The PCR program was further modified by introducing an additional step with three cycles, at a higher annealing temperature and the spurious amplification could be suppressed (Fig.4).

DISCUSSION

Efficiency of PCR reflects the capability of a primer pair to produce faithful amplification, which is close to the theoretical doubling of target sequence per cycle. Ill designed primers allow co-amplification of unintended regions compromising both specificity and efficiency. The principles of PCR primer design were reviewed by Singh and Kumar (2001). However in many cases, whole genome sequence is not available in public domain which makes the researcher to choose primers without the knowledge of other probable targets.

The annealing temperature (T_a) used for primer- template interaction is a function of T_m. The annealing temperature occurs within a range of 4-10°C lower than T_m and increased T_a in PCR results in poor or no amplification (Wu *et.al.*, 1991). In case of both FUT1 and HSD 17β, the

annealing temperature was observed to be within this range. It implies that the nearest neighbour thermodynamics (Santa Lucia.,1998) can efficiently predict the T_m. Lorenz (2012) suggested that the melting temperature of the primers should range between 45-65°C with the window between 52-58°C being the optimum. However T_m for the primer pair should not differ by more than 5°C. Dieffenbach *et.al*., (1993) were of the opinion that PCR primers should maintain a reasonable GC content. Oligonucleotides which were 20 bases long with a 50percent G + C content generally had Tm values in the range of 56-62°C. Primers for both the loci were within the length limit and had acceptable T_m. Even with GC content in excess of 50 percent, spurious amplicons had appeared in the PCR for FUT1 and HSD 17β loci. The high GC content is reflected in the elevated T_m of the primers. The factor that the length of primers for HSD 17β being slightly less (18 and 19 bases) than the optimum, could also have favoured mispriming.

The concentration of $MgCl_2$, dNTP's and *Taq* polymerase also influence the occurrence of spurious amplicons. Higher concentration of $MgCl_2$, dNTP and *Taq* polymerase is known to favour mispriming and generation of unintended products. The recommended concentration of $MgCl_2$ varies between 0.5 to 5.0 mM. In this experiment, a concentration of 2.5mM $MgCl_2$ was used which is well within this range.

Sarkar *et.al*. (1990) used formamide to prevent spurious amplification in PCR, which employed primers with GC content in excess of 55 percent. They had further observed that in case of primers with high GC content, formamide added to the reaction mixture at 1.5 to 5 percent levels prevented spurious amplification. In the present study, formamide included at 0.75 percent V/V, was sufficient to banish non specificity.

Inclusion of a separate cycle with three steps (steps 2 to 4, Table: 2) to amplify HSD 17β comes from 'Touchdown' programme (Don *et.al*., 1991). The touchdown programme is widely used to amplify repetitive sequences, like microsatellite markers which generate lot of noise. In order to overcome spurious priming, few initial cycles are run at higher annealing temperature thereby enhancing the specificity of primer binding. Addition of a high temperature step in this experiment possibly increased the specificity of PCR.

Innis *et.al*. (1988) observed that *Taq* polymerase added nucleotides at a rate of about 60 nucleotides per second. Some other reports suggest the speed to be between 35 and 100 nucleotides per second. The rule of the thumb is to provide extension time of one minute per Kb size of the product. In current study, reduction of extension time also played a part in blocking spurious amplifications in both FUT1 and HSD 17β loci.

CONCLUSION

Non specific amplification in the case of primers with high GC content could be averted by using formamide and adjustment of annealing and extension time and introduction of a high temperature step so as to favour the formation of the product of interest.

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