SHORT COMMUNICATIONS

Effect of heat denaturation of target DNA on the PCR amplification

(Recombinant DNA; polymerase chain reaction; Aeromonas salmonicida; product yield)

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SUMMARY

The polymerase chain reaction (PCR) and amplification of specific regions of DNA in vitro is a widely used and powerful technique, and the optimization of conditions used to maximize PCR product yield has received much attention. We have shown that lengthy denaturation times of template DNA ranging from 1 to 7 min at pH 7.0-8.0, that are often employed prior to the start of a PCR reaction, result in marked degradation of the template. This can result in a significant reduction in the yield of PCR products larger than 500 bp, by up to 99%. This effect was demonstrated for both complex genomic template DNA, and also for a 2691-bp linear piece of template DNA using both a rapid hot-air thermocycler and a conventional block thermocycler. This decrease in product yield is likely due to the increased degradation of the template or target DNA as a result of pre-amplification denaturation (PAD). We therefore recommend that when amplifying larger pieces of DNA, the template DNA should not be exposed to PAD prior to a PCR reaction, irrespective of the starting pH of the template solution.

INTRODUCTION

The use of PCR (Saiki et al., 1985) to amplify and clone a single-copy gene from a complex chromosome remains one of the primary uses of this powerful technique. Typically, PCR reactions involve the heat denaturation of the double-stranded template or target DNA prior to cycling and amplification. The use of hot air thermocyclers is becoming increasingly popular, especially for diagnostic purposes, as they are far more efficient in terms of time and use of expensive materials than their conventional ‘block cycle’ counterparts. It has been reported that denaturation of supercoiled genomic DNA is necessary for PCR amplification to occur (Wittwer and Garling, 1989; Bej et al., 1991; Hammar et al., 1992). Indeed, plasmid template DNA is often denatured prior to amplification. The aim of this study was to investigate the effect on PCR product yield of denaturing template DNA prior to commencement of amplification. We demonstrate that heat-denaturation of template DNA prior to the start of the cycling reaction is not only unnecessary, but can significantly decrease the yield of larger DNA fragments.

EXPERIMENTAL AND DISCUSSION

(a) Oligodeoxynucleotide primer pairs

Primer pairs AP-1/AP-2, RAA2/AP-2 and RAA2/RAA3 (Fig 1) within and flanking the gene encoding the 50778 Da surface array protein of Aeromonas salmonicida (Chu et al., 1991) were chosen which would
produce 421-bp, 1944-bp and 2691-bp fragments of DNA from the *A. salmonicida* chromosome, respectively. The \% (G+C) of these pieces was 48.9%, 47.4% and 46.7%, respectively, compared to the \% (G+C) content of the entire *Aeromonas* genome (Belland and Trust, 1988). The genomic DNA of *A. salmonicida* A450 prepared as described previously (Manning et al., 1986) was used as the template DNA.

(b) Effect of PAD using genomic template

The A450 template DNA in 1 x TE was diluted to 50 ng/μl in sterile distilled water, and subjected to PAD in a separate tube at 95°C for 1, 3, 5 and 7 min, chilled immediately on ice, and then added to the amplification mixture. The pH of the resultant amplification mixture was 8.0–8.1, which represents the preferred pH for Taq enzyme activity and amplification. The consequences of the template denaturation are shown in Fig. 2. The 421-bp product was amplified irrespective of the length of heat treatment to the template DNA (Fig. 2A). In contrast, the longer the template DNA was held at the denaturation temperature before cycling began, the lower the product yield, until no product was detectable (a decrease of 99% as measured by densitometry) for both the 1944-bp fragment at 7 min and the 2691-bp fragment at 3 min (Fig. 2A and 2B, respectively). The product yields of these two larger fragments were significantly greater when the template DNA was not heat-denatured prior to amplification (0 min in Fig. 2A, 2B).

This effect of PAD on amplification of these fragments was also tested in a conventional PCR block thermocycler (ERICOMP Inc., SingleBlock™ System) to eliminate the possibility of an artifact caused by the use of the air thermocycler. The cycle parameters commonly employed in block thermocyclers that held the sample at the denaturation and annealing temperature for only 1 s, the yield of the 2691-bp product was increased 2.6-fold, as measured by densitometry when no PAD was employed (data not shown), and the rate of decrease in product yield, when PAD was employed, paralleled that seen previously with the air thermocycler (Fig. 2C). We consider that the extended denaturation step during every PCR cycle commonly employed in block thermocyclers may not only be unnecessary to denature template DNA, but also causes a significant reduction in product yield by the end of 25–35 cycles, compared to the brief denaturation time of 1 s used in this study. This may be due to the half-life of the AmpliTaq polymerase being 40 min at 95°C, or the degradative effect of elevated temperatures on template DNA. In corroboration of these results, it has been reported that improved PCR product yields have been attained with lower denaturation temperatures during cycling (Yap and McGee, 1991).
(c) Effect of PAD using short, linear template

Many researchers commonly use either circular or linearized plasmid DNA as a template. To determine whether the effect of extended PAD occurred only in chromosomal samples or was an effect caused by the particular method by which genomic DNA was isolated, we tested the effect of PAD using the 2691-bp PCR product as template DNA in the air thermocycler. The 2961-bp product initially generated by PCR amplification, at pH 8.0, was subjected to PAD for increasing times, and then 5 ng was used as template DNA for further amplification. A decrease in product yield was observed after 1 min of PAD (Fig. 3), however, the rate of decrease was less significant than with the chromosomal template, such that after 5 min of PAD, a small amount of product was still apparent (Fig. 3).

The possibility existed that the initial pH of the template DNA when exposed to PAD was crucial to the rate of decrease in product yield, as low pH is known to depurinate DNA, and thus cause more significant degradation, especially at elevated temperatures. If genomic A450 DNA in 1 x TE (pH 8.0) was exposed to PAD for increasing periods of time, and subsequently diluted in distilled water to 50 ng/µl, the decrease in product yield was similar to that seen when using the 2691-bp PCR product (pH 8.0) as template DNA (data not shown). The difference in rate of decrease in PCR product yield detected with the two separate methods of genomic template preparation, that is dilution before or after PAD, was presumably due to the lower pH of the diluted DNA during PAD, measured to be pH 7.0.

(d) Conclusions

(1) The basis for the results presented here is that the template DNA is degraded when held at the denaturation temperature for long periods. Indeed, agarose gel electrophoresis of unamplified chromosomal DNA at pH 8.0 that had been exposed to heat denaturation at 95°C for increasing times showed marked degradation correlating with the length of heat denaturation (Fig. 4), and heat denaturation of DNA at pH 6.0 and pH 7.0 displayed similar degradation (data not shown). Such degradation would statistically favour breakage between the priming sites for larger PCR products over smaller products. This hypothesis correlates with data seen in Fig. 2, where the smallest 421-bp product is still produced with high yield even after 7 min PAD, as compared to the larger fragments.

(2) There is a direct correlation between template pH during PAD and decrease in yield of large PCR products. A more significant decrease in product yield is observed when the template DNA is diluted in distilled water prior to PAD, due to a lower pH. Moreover, attempting to maintain a stable pH by diluting template DNA in 1 x TE (pH 8.0) is not recommended due to the chelating effect of EDTA on Mg2+ ions required for Taq polymerase activity. However, as we have shown it to be unnecessary to denature template DNA prior to amplification, these problems can be easily circumvented.

(3) The effect of decreased product yield due to PAD described here is not restricted to Aeromonas DNA. We have found the same effect during amplification of DNA from Escherichia coli and Campylobacter spp. We conclude that the length of template denaturation time before and during cycling should be included as an important variable during optimization of maximal product yield for each individual PCR application irrespective of the source or pH of template DNA or type of PCR thermocycler being used.

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REFERENCES
