

Eliminating primers from completed polymerase chain reactions with exonuclease VII

Honghua Li⁺, Xiangfeng Cui and Norman Arnheim*

Molecular Biology, University of Southern California, Los Angeles, CA 90089-1340, USA

Received December 3, 1990; Revised and Accepted May 10, 1991

ABSTRACT

Single-stranded oligonucleotide primers can be efficiently removed after PCR using E.coli exonuclease VII. Even only a few molecules of double stranded PCR product are unaffected by a treatment which eliminates 20 picomoles of primer in the presence of 500 ng of denatured genomic DNA. Exonuclease VII treatment is rapid and could simplify complicated multistep PCR protocols.

INTRODUCTION

For some applications of the polymerase chain reaction (PCR, 1,2,3) several successive amplification steps are involved. To minimize the effect of primers left over from previous steps on subsequent reactions in which different primers are used, product can be physically separated from primer by a number of standard procedures. Alternatively a small aliquot (1–3 μ l) of PCR product can be diluted in a new reaction mix. In this paper we show that PCR primers can be removed efficiently from a completed reaction by a simple E.coli exonuclease VII (exo VII) digestion step. Exo VII is ideal for this purpose because of its specificity for single stranded DNA (4–6). It initiates hydrolysis from both the 5' and 3' ends in a processive fashion eventually yielding short acid-soluble oligonucleotides (4,5). We found that exo VII is highly active in the commonly used 1 \times PCR buffers and therefore tested whether simple addition of the enzyme to the sample after PCR could provide a rapid way to eliminate primers.

MATERIALS AND METHODS

Exo VII was purchased from GIBCO-BRL and Taq polymerase obtained from Perkin-Elmer Cetus Corporation. PCR and exo VII digestion was carried out in 50 μ l of 1 \times standard PCR buffer (Perkin-Elmer Cetus) containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl₂ and 100 μ g/ml gelatin. Primers TaM2 (GATCTCTT-CCTGGGAAGAAG) and TaM3 (GATACCTGCAAAAGACATGG) amplify a 196 base pair fragment of the human parathyroid hormone gene (PTH; 7). TaM5 (TC-CCATTAGCTCCCCACTTC), was used as a nested primer and

amplifies a 157 base pair fragment with the primer TaM3 (Figure 1). Sperm samples were prepared by staining the sperm heads with Hoechst 33342 and sorting into 96 well microtiter dishes with flow cytometry as described (8) and then lysed (9). Exo VII was added directly to the PCR sample tube and digestion carried out at 37°C for 30 minutes. The enzyme was inactivated by incubating the samples at 95°C for 10 minutes. Three different protocols were used for PCR. Protocol 1: 95°C, 15" denaturation; 60°C, 15" annealing and 72°C, 30" extension. Protocol 2: 92°C, 30", denaturation; 60°C, 5' annealing and extension for two cycles but with the second cycle ending in a 1' 72°C incubation. Protocol 3: 95°C, 30" denaturation; 60°C 3' annealing and extension for 10 cycles, and 60°C, 2' annealing and extension for an additional 30 cycles. PCR products were analyzed on 8% polyacrylamide gels and stained with ethidium bromide.

RESULTS

Eliminating PCR primers by exo VII digestion

The first experiment was designed to test whether exo VII could eliminate PCR primers in the presence of a large amount of double stranded DNA. Four samples containing 10 pmoles each of primers TaM2 and TaM3 together with 556 ng human genomic DNA were digested with 1 (samples 3 and 4) or 2 (samples 5 and 6) units of exo VII. To determine the degree of primer elimination and the integrity of the genomic DNA, TaM2 and TaM3 were added back to samples 4 and 6 after exo VII inactivation and 25 cycles of PCR were carried out on all four samples using Protocol 1. The correct PCR products were observed (Figure 2) in the samples with new primers added (lanes 4 and 6) while no product was detected in the absence of newly added primers (lanes 3 and 5). The amount of specific product detected was about the same as in a control tube which was not treated with exo VII (lane 2) indicating that the double stranded genomic DNA was not significantly damaged by the enzyme treatment. When 50 pmole of each primer and 1 unit of exo VII were used, amplification was detected in the absence of primer addition (lane 9) but not if 2 units of enzyme were used (lane

* To whom correspondence should be addressed

⁺ Present address: Division of Biology, California Institute of Technology, 139-74, Pasadena, CA 91125, USA

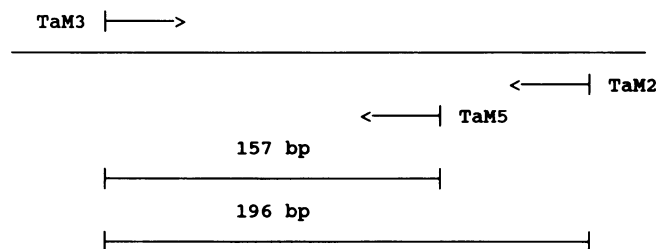


Figure 1. Relative positions of the PTH primers used for PCR. The name of each primer and its orientation is given.

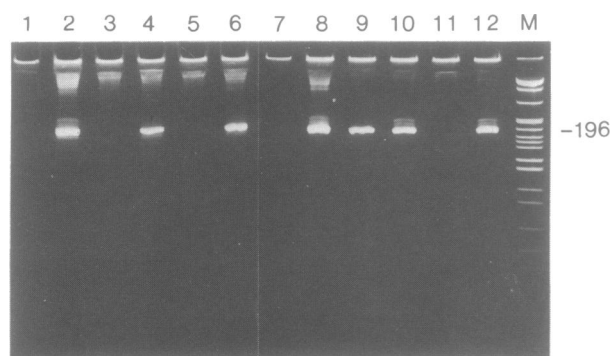


Figure 2. Effect of PCR on the digestion of primers TaM2 and TaM3 by *exo VII*. Following the addition of the reagents and incubations where necessary, PCR was carried out using Protocol 1. The expected product was 196 bp. Each sample contained 556 ng of undenatured human genomic DNA except for lanes 1 and 7. P = picomoles of each primer (TaM2 and TaM3) originally added; E = units of *exo VII* added; A = picomoles of each primer (TaM2 and TaM3) added after *exo VII* digestion. 1, P(10) without human genomic DNA. 2, P(10). 3, P(10)+E(1). 4, P(10)+E(1)+A(10). 5, P(10)+E(2). 6, P(10)+E(2)+A(10). 7-12, the same as 1-6 respectively with the exception that P = 50 and A = 50. M = molecular weight marker; pBR322 digested with *Msp I*.

11). The short oligonucleotide products of *exo VII* (4,5) apparently did not interfere with PCR when new primers were added.

The effect of *exo VII* on limiting amounts of double stranded DNA

The experiment described above suggested that the double stranded target present in 556 ng of human DNA was not damaged significantly by *exo VII*. To further test *exo VII*'s specificity for single stranded DNA, samples with 1 or 5 human sperm heads were subjected to 2 PCR cycles (Protocol 2) with 10 pmoles each of TaM2 and TaM3 to convert the target fragments (which were denatured as a result of sperm lysis by KOH) into double-stranded form. This yielded theoretically 4 or 20 copies of the target fragment in the single and 5 sperm samples, respectively. Each first round sample was then digested with 1.5 units of *exo VII* and subjected to a second round of 40 PCR cycles (Protocol 3) with or without primers TaM2 and TaM3. Finally a 1 μ l aliquot from each sample and control was further amplified for an additional 25 cycles (Protocol 1) with primers TaM3 and TaM5. The results are shown in Figure 3. The digested samples which did not have primer added for the second round, (1 sperm, lanes 4-6; 5 sperm, lane 11) gave no specific PCR product after the third round. However, the digested

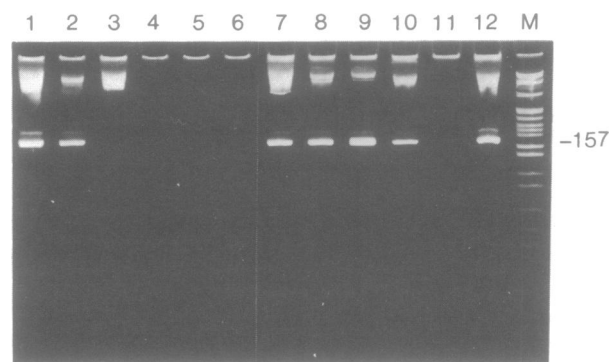


Figure 3. The effect of *exo VII* digestion on a few double stranded DNA molecules. Each sample initially contained 10 picomoles each of TaM2 and TaM3. E = units of *exo VII* added; S = number of sperm added; P = micromolar concentration of each primer (TaM2 and TaM3) originally added; A = micromolar concentration of each primer (TaM2 and TaM3) added after *exo VII* digestion. 1-3, S(1). 4-6, S(1)+E(1.5). 7-9, S(1)+E(1.5)+A(10). 10, S(5). 11, S(5)+E(1.5). 12, S(5)+E(1.5)+A(10). See the text for the details of the PCR protocol.

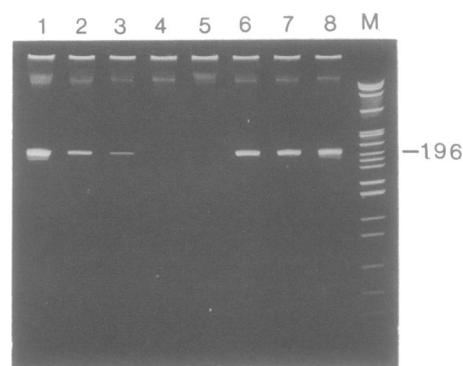


Figure 4. The effect of denatured genomic DNA on primer digestion by *exo VII*. In all amplifications protocol 1 was used. All samples contained 500 ng of human DNA and 10 picomoles of each primer. Lane 1, 30 cycles of PCR. Lanes 2-4, *exo VII* digestion with 1, 2 or 4 units of enzyme, respectively, after 15 cycles of PCR. Following enzyme inactivation, amplification was continued with an additional 15 cycles. Lanes 6-8, same as lanes 2-4 except that 10 picomoles of each primer was added to the samples after *exo VII* treatment. Lane 5, 15 cycles of PCR with 500 ng of genomic DNA.

samples which were amplified in the second round in the presence of primers (1 sperm, lanes 7-9; 5 sperm, lane 12) showed a clear band of the expected size. The control samples without *exo VII* digestion are also shown (1 sperm, lanes 1-3; five sperm, lane 10). In one of the single sperm control samples no specific band was detected (lane 3). These results indicated that 1.5 units of *exo VII* did not significantly digest a few double stranded DNA molecules while at the same time functionally eliminating 20 pmoles of single-stranded primers. This specificity for single stranded DNA further confirms earlier studies characterizing this enzyme (4,5).

Primer digestion in the presence of denatured genomic DNA

As shown above, *exo VII* digestion of single stranded primers is highly efficient in the presence of a large amount of double stranded DNA and even small amounts of double stranded PCR product are resistant to digestion. In many applications of PCR where genomic DNA is used as template, denaturation during

the course of the reaction results in a relatively large amount of single stranded DNA which can compete with primer as substrate for *exo VII*. We tested for primer elimination under these conditions. As shown in Figure 4, when PCR was carried out for 30 cycles (Protocol 1) on 500 ng of human genomic DNA using 10 picomoles of each primer a major band of product was detected (lane 1). In lanes 2–4 three identical samples were amplified for 15 cycles followed by *exo VII* digestion with 1, 2 or 4 units of enzyme respectively. After inactivating the *exo VII*, the samples were then amplified for an additional 15 cycles. With 4 units of enzyme (lane 4) only a trace amount of product was observed after a total of 30 cycles. The amount of product was very close to that observed when 500 ng of genomic DNA was amplified for only 15 cycles (lane 5) and indicates that primer digestion was virtually complete. If fresh primers were added after *exo VII* digestion and enzyme inactivation, the expected amount of product was observed after an additional 15 cycles, confirming again that the double stranded PCR product was resistant to digestion (Lanes 6–8).

DISCUSSION

One of the major advantages of using *exo VII* to eliminate PCR primers is that only enzyme addition and incubation steps are involved. This could be useful if many samples need to be processed and would facilitate automation. Both the data we present in this paper as well as unpublished experiments indicate that 4–8 units of the enzyme would be required for eliminating 20–100 picomoles of primer (20-mers) in samples containing on the order of 500 ng of denatured human genomic DNA. However the relationship between the number of units of *exo VII* required to digest known amounts of primer and genomic DNA should be established empirically for each application.

In the paper by Mueller and Wold (10) a very useful procedure was described for detecting protein binding sites on DNA molecules *in vivo*. This method involves an initial primer extension, PCR with a distinct set of primers followed by primer extension with yet again a new primer. The method requires great care in the selection of primer melting temperature since primers from an earlier step can compete with newly added ones. This requirement might be eliminated by using *exo VII*. In conclusion, because of its specificity for single stranded DNA, *exo VII* can be used for the removal of single stranded PCR primers. This method may aid in the development of simpler and more rapid multiple step PCR procedures.

REFERENCES

1. Saiki, R., Scharf, S., Faloona, F., Mullis, K., Horn, G., Erlich, H., and Arnheim, N., (1985) *Science*, 230, 1350–1354.
2. Mullis, K. B., and Faloona, F. A. (1987) *Methods Enzymol.*, 155, 335–351.
3. Saiki R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., and Erlich, H. A. (1988) *Science*, 239, 487–491.
4. Chase, J.W. and Richardson, C.C. (1974a) *J. Biol. Chem.*, 249, 4545–4552.
5. Chase, J.W. and Richardson, C.C. (1974b) *J. Biol. Chem.*, 249, 4553–4561.
6. Chase, J.W. and Vales, L.D. (1981) in *Gene Amplification and Analysis* (Chirikjian, J.G. and Papas, T.S., eds.) Vol.2, p.148, Elsevier, New York.
7. Li, H., Cui, X. and Arnheim, N. (1990) *Proc. Natl. Acad. Sci.*, 87, 4580–4584.
8. Li, H., Cui, X., and Arnheim, N. (1991) *Methods-A Companion to Methods in Enzymology* In Press.
9. Cui, X., Li, H., Goradia, T.M., Lange, K., Kazazian, H.H., Galas, D.J. and Arnheim, N. (1989) *Proc. Natl. Acad. Sci. USA.*, 86, 9389–9393.
10. Mueller, P. R. and Wold, B. *Science*, 246, 780–786.