Automated DNA Sequencing Requiring No DNA Template Purification


ABSTRACT

A critical component in automated fluorescent DNA sequencing is good quality of DNA template. In an effort to reduce the dependence of sequencing success on DNA template quality, the effect of heat-soaked PCR on automated sequencing reactions has been examined. We have found that the heat-soaked PCR protocol considerably improves the overall quality of sequence data and significantly reduces the dependence on the quality of DNA templates. The improvement is corroborated by our ability to obtain over 500 bp of readable sequence per reaction using DNA from E. coli lysates as template obviating DNA purification.

INTRODUCTION

Our laboratory has recently identified and characterized a spinal muscular atrophy (SMA) candidate gene mapping to chromosome 5q13. We are currently sequencing the 154-kb PAC clone 125D9, which contains the entire candidate SMA gene (8). For this large-scale sequencing project, partial Sau3AI, (selected for 5-kb insert size), EcoRI and HindIII libraries have been prepared for use in an ordered shotgun sequencing strategy as described by Chen et al. (3). In addition, transposon-enhanced sequencing using the TN1000™ kit (Gold BioTechnology, St. Louis, MO, USA) has been used. The resulting subclones are sequenced using the Applied Biosystems Models 373A and 373 Automated Sequencers (Perkin-Elmer/Applied Biosystems Division [PE/ABI], Foster City, CA, USA).

Optimization of DNA sequencing methodology should increase laboratory throughput and facilitate large-scale genome sequencing. Automated fluorescent DNA sequencing relies upon good quality of DNA template for success, the importance of which is reflected in the stringent recommendations by manufacturers and users of automated sequencers in template preparation procedures. In most cases, poor quality of sequence data is a result of inadequate DNA template preparation. In an effort to reduce the dependence of sequence quality on DNA template, we have investigated the effect of heat-soaked polymerase chain reaction (HS-PCR; Reference 9) on automated DNA sequencing reactions. Our results suggest that the HS-PCR protocol significantly reduces nonspecific background signal. Our ability to sequence DNA from E. coli cell lysates was then explored. Manual sequencing of plasmid DNA isolated directly from single plaques or colonies by lysis in distilled water (6) and in proteinase K (10) has been described. Using these approaches, sequences of approximately 200 bp (6) and up to 300 bp (10) per reaction, respectively, have been achieved. However, automated sequencing using bacterial cell lysates as template has not yet been reported. Herein, we report that the use of HS-PCR in automated DNA sequencing reactions obviates DNA template purification while still providing over 500 bp of readable sequence per reaction with almost insignificant background.

MATERIALS AND METHODS

The primary sequencing method used in our laboratory has been the manufacturer’s recommended protocol (1,2) accompanying the PRISM™ Dye Terminator Cycle Sequencing Core Kit [PE/ABI] with AmpliTaq® DNA polymerase, FS (fluorescent sequencing; Perkin-Elmer, Norwalk, CT, USA). Purified plasmid DNA, PCR fragments or plasmid-containing E. coli cell lysates were used as DNA sequencing templates. The plasmid vectors used for the experiments were pUC18 and Bluescript® II SK (-) (Stratagene, La Jolla, CA, USA). Plasmid DNA and PCR fragments were purified as described in the PE/ABI manual provided with Taq DyeDeoxy™ Terminator Cycle Sequencing Kit (Part No. 901497). To prepare cell lysates (4), E. coli cells DH5α or BW26 (Gold Biotechnology)
Short Technical Reports

lines containing plasmids were grown in LB medium with appropriate antibiotics at 37°C for 20 h. The overnight cultures (1.5 mL) were pelleted, resuspended in 200 µL of dH2O containing 50 µg/mL proteinase K (Boehringer Mannheim, Quebec City, Quebec), placed at room temperature for 2 h or at 4°C overnight and incubated on a 50°C heating block for 30 min. No buffer was needed in the proteinase K digestion, thereby removing a factor that might interfere with the sequencing reactions. The mixture was heated at 95°C for 15 min and centrifuged at 12 000×g for 2 min. The supernatant was used in two different cycle sequencing protocols in a Model 9600 PCR System Thermal Cycler. The first protocol was as described by the PE/ABI Dye Terminator Cycle Sequencing Kit’s manual (2). In the second protocol, HS-PCR, described previously by Ruano (9), was introduced into our sequencing reaction. Briefly, 0.5 µg of purified DNA template in 11 µL of distilled water or 11 µL of E. coli cell lysate was mixed with 4 µL of TACS buffer [400 mM Tris-HCl, 10 mM MgCl2, 100 mM (NH4)2SO4, pH 9.0] in a 0.2-mL MicroAmp™ reaction tube (Perkin-Elmer) and then heated to 94°C for 30 min in a Model 9600 PCR System Thermal Cycler. At the conclusion of the incubation, 5 µL of sequencing reagents at 4× concentration were added into the preheated reacting tubes left in the thermal cycler at 94°C (90°C if handling more than 24 samples), and PCR cycling immediately begun (30 cycles; 96°C for 10 s; 50°C for 5 s; 60°C for 4 min). The addition of the reagents should be carried out as quickly as possible to avoid prolonged heating of the polymerase. A premixture, which contained 50 µL each of G, A, T, C DyeDeoxy Terminators, 100 µL of dNTP mixture (concentrations of the terminators and dNTP mixture unavailable from the manufacturer) and

Figure 1. Comparison of PE/ABI protocol and heat-soaked protocol. Approximately 0.5 µg of purified plasmid DNA (3A8, a clone from the PAC 125D9 partial Sau3AI library) was sequenced with M13 forward primer using the ABI PRISM Dye Terminator Cycle Sequencing Core Kit with AmpliTaq DNA Polymerase, FS. (A) PE/ABI protocol. (B) Heat-soaked PCR protocol. The samples were analyzed on a Model 373A Automated Sequencer using a 6.0% polyacrylamide gel. Nucleotide 90 in sequence A corresponds to nucleotide 100 in sequence B.
100-µL of AmpliTaq DNA polymerase, FS (8 U/µL), was routinely prepared in a large volume and stored at -20°C. The 5 µL of sequencing reagents were then prepared by adding 1 µL of any sequencing primer (0.1 µg/µL) to 4 µL of the premixture before the sequencing reaction. Primers tested with the protocol included T7, T3, M13 forward, M13 reverse and more than 50 oligonucleotides (20–25-mer) synthesized in our laboratory. Purification of sequencing products, preparation of 6% polyacrylamide gel, electrophoresis, data collection and analysis were carried out as outlined in the PE/ABI manual (1). The preparation of 34-cm, 5.5% polyacrylamide stretch gel for PE/ABI Model 373 was as described by McDonald et al. (7). The conjugal transfer of a plasmid to introduce TN1000 insertions into a target sequence was carried out as described by the supplier (Gold BioTechnology).

RESULTS AND DISCUSSION

In an effort to both increase sequencing efficiency and data quality, the effect of HS-PCR on automated sequencing reactions utilizing purified plasmid DNA or PCR fragments as template was studied. When compared to the protocols recommended by the manufacturer (PE/ABI), the HS-PCR protocol consistently reduced the level of background signal (Figure 1). In the usual protocol, DNA mixed with reagents at room temperature could permit nicked, sheared or single-stranded DNA to serve as a template for nonselective priming and extension during the initial ramp from room temperature to 96°C, by partial Taq DNA polymerase activity (1%–40%) in the 25°–55°C range (5). We believed that such nonselective priming and extension may be one of the most common causes of background noise, obscuring the true sequence. In the HS-PCR...
protocol, annealing of the completely denatured template and the primer occurred under high and stringent temperature minimizing nonspecific priming and extension. As a result, both the signal-to-noise ratio and the length of legible sequence appeared to be significantly improved. A second common cause of significant noise and signal attenuation may be the contamination of template DNA with impurities. The inhibition of Taq DNA polymerase activity by these putative contaminants may lead to inefficient DNA synthesis, shorter chain extension and false incorporation of dye terminators. It is conceivable that this problem may be rectified by degradation of inhibitory substances during the 30-min incubation at 94°C. Regardless of the precise mechanism, a genuine reduction in nonspecific background signal was observed consistently, permitting longer legible sequences.

Given the improvement in sequencing by the HS-PCR protocol using standard purified templates, sequencing of DNA directly from E. coli lysates was attempted, resulting in legible sequence lengths of over 500 bases (Figure 2). To date, more than 800 samples have been successfully sequenced using E. coli lysates as templates. These results underscore the potential utility and versatility of the HS-PCR adaptation, especially since DNA template preparation from crude cell lysates is rapid and straightforward. Clearly, this approach should result in significant savings in time and cost, particularly for large-scale sequencing projects. It may also prove to be helpful in the automation of mutation screening in diagnostic laboratories using blood samples without DNA purification since, although clearly not a factor in the present case, haemoglobin is one example of inhibitor of PCR that can be effectively inactivated by boiling (9).

REFERENCES


This project was supported by grants from the Muscular Dystrophy Association (USA) and the Networks of Centres of Excellence (Canadian Genetic Diseases Network). We thank Xiaolin Kang, Stephen Baird and Jian Ying Xuan for technical assistance and helpful discussion. A.M. and R.G.K. are Medical Research Council (Canada) Scientists. Address correspondence to Qianfa Chen, Molecular Genetics Laboratory, Children’s Hospital of Eastern Ontario, 401 Smyth, Ottawa, ON, Canada K1H 8L1. Internet: qianfa@mcheo.med.uottawa.ca

Received 24 January 1996; accepted 8 May 1996.

Qianfa Chen, Catherine Neville, Alex MacKenzie and Robert G. Korneluk
Children’s Hospital of Eastern Ontario
Ottawa, ON, Canada