A Rapid Droplet Method for Sanger Dideoxy Sequencing

ABSTRACT

A method for performing the dideoxy sequence reaction on petri dishes is described. It allows rapid manipulation of clones and provides large amounts of sequence information quickly and without the need for elaborate laboratory equipment.

INTRODUCTION

The Sanger dideoxy chain terminating method is a widely used DNA sequencing procedure (8,9). It allows the generation of 200-400 base pairs of sequence per template with little effort. With the use of gradient polyacrylamide gels and [^{35}S]dATP, this range can be extended to 400-500 base pairs of sequence per clone. Over the past ten years there have been numerous modification and refined sequencing strategies which have added to the speed of generating sequence information (11).

We have added several advantageous features to the chain terminating method that allows rapid sequencing of DNA. The method eliminates Eppendorf tubes and hence centrifugation steps can be omitted. All reactions and manipulations are performed directly on petri dishes with 8 clones per plate; larger numbers of samples can be accommodated on additional plates. This method is applicable to both ^{35}S and ^{32}P label. With the use of 48 lanes per standard sequencing gel (20 cm x 40 cm) this procedure will enable a researcher to collect over 250 nucleotides of data for each clone using a single sequencing gel, and a larger number of clones can be analyzed simultaneously.

MATERIALS AND METHODS

DNA polymerase large fragment (Klenow) was purchased from Promega Biotech. Dideoxynucleotides and deoxynucleotides were obtained from Pharmacia Biochemicals. The radiolabeled nucleotides (^{32}P and ^{35}S α-P-labeled dATP) were supplied by Amersham International. The Hamilton dispenser was bought through CanLab. Oligodeoxynucleotide primers were synthesized on an Applied Biosystems model 380A DNA synthesizer, deprotected and purified as described (1).

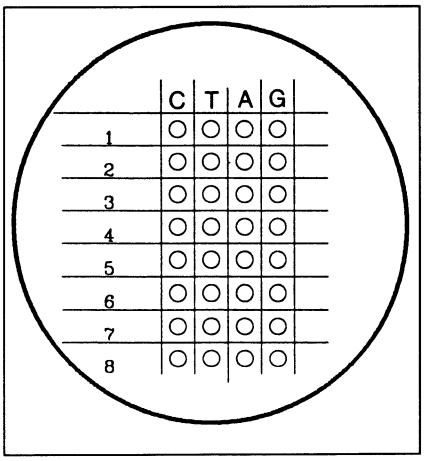

Figure 1. Schematic representation of the grid arrangement of droplets on a petri plate. After annealing of primer to template, the mix is divided into four to represent each of the four termination reactions (C, T, A, G).

Sequencing Protocol

Isolation of pEMBL and M13 single stranded DNA was achieved using standard procedures described by Ner et al. (7). Standard protocols for the isolation of plasmid DNA were used (5) and the procedure of Chen and Seeburg (2) was used to prepare the DNA for double stranded sequencing (10). Solutions for chain terminator sequencing were those described by Sanger et al. (8). All reactions were performed in droplets on the surface of an 8.5 cm plastic petri dish (Figure 1), and as many as 8 template DNAs could be sequenced in a single dish. A moistened and rolled tissue was placed inside the perimeter of the dish and the lid replaced during incubations to prevent sample evaporation. The oligonucleotide primer (0.3 pmol in 2 µl of 3X annealing buffer) was added to the single stranded DNA solution (0.3 pmol in 5 µl TE) followed by annealing for 5 min at 65° C by floating the covered dish on the surface of a waterbath. After cooling for 5 min at room temperature, 2 µl of each of the four solutions corresponding to each of the dideoxynucleotide-deoxynucleotides were placed in rows beside the DNA-containing droplet. To each DNA-containing droplet, 4 µl of enzyme/label solution (6 µl H_2O, 1 µl 10X low salt restriction buffer, 1 µl 12 µM dATP, 1 µl (5 units) Klenow fragment, and 2 µl [α-^{35}S]dATP (>1000 Ci/mmole)) were added and mixed with a pipette tip. Then 2 µl of this mixture was added to each dideoxynucleotide-deoxynucleotide droplet. After incubation at 37° C by floating the covered dish on a waterbath for 15 min the reactions were chased with 2 µl of 1 mM dNTP solution and the dish returned to 37° C for 15 min. Reactions were stopped by addition of 2 µl of a formamide/dye/ EDTA solution, and samples denatured by floating the dish (without lid to promote evaporation and sample concentration) on a 95° C waterbath for 3 min. Approximately 2 µl of each reaction was loaded onto a

48 lane, 8% polyacrylamide - 8 M urea, wedge shaped gel (0.35 to 0.7 mm). After electrophoresis for approximately 2 h at 1500 V the urea was removed from the gel by soaking for 30 min in 10% acetic acid, and the gel was dried and autoradiographed (24 h) without an intensifying screen.

RESULTS

We present a very simple and quick method for sequencing a large number of templates using the dideoxy chain terminating method. The method requires very simple apparatus and minimizes costs by eliminating the need for centrifuges, Eppendorf tubes and adaptors. In addition, performing the reactions on the petri dishes prevents confusion (due to mixing of tubes) and allows easy handling of radioactive materials. The reaction components are added using a 2 µl Hamilton dispenser. Cross contamination is avoided by preforming a droplet on the tip of the dispenser. The droplet is then transferred to the petri dish by gently allowing the solution to come into contact with the droplet already on the plate. On completion of the reaction the samples are denatured on the petri dish by floating the dish on a 95° C waterbath. This has the additional advantages of concentrating the sample to a smaller volume (compatible with the use of 48 lane sequencing gels) and allows samples to be loaded into the wells quickly and efficiently using a drawn out capillary tube.

The autoradiographs in Figure 2 are of a single stranded pEMBL template bearing the coding sequence of the yeast cytochrome *c* peroxidase gene (3), sequenced using [α-^{35}S]dATP. At least 250 nucleotides of sequence from the priming site can be read. With longer electrophoretic runs, this can be extended to over 400 nucleotides. Thus, from 12 clones, sequence information of over 4.5 kb can be obtained using a combination of a short time and a long time gel electrophoresis. The use of [α-^{32}P]dATP will also yield similar results. Figure 3 shows a portion of the coding sequence of cytochrome *c* peroxidase sequenced with [α-^{32}P] dATP using alkaline denaturation of

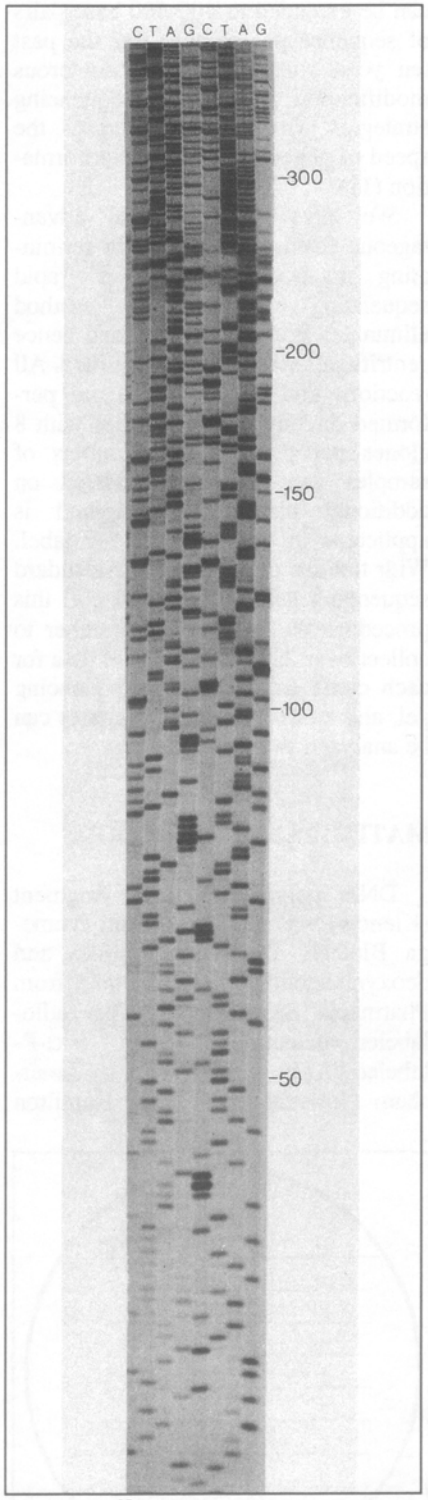

Figure 2. [α-^{35}S]dATP dideoxy sequencing of a single stranded DNA template of the cytochrome *c* peroxidase gene cloned in pEMBL8+. The coding region is sequenced with two oligonucleotide primers. The reactions were performed as described in the text and electrophoresed on an 8% polyacrylamide wedge shaped gel (48 lanes) at 1500 volts for 1.5 h. An overnight exposure of the gel allows 250-300 nucleotides to be read from this short electrophoresis run.

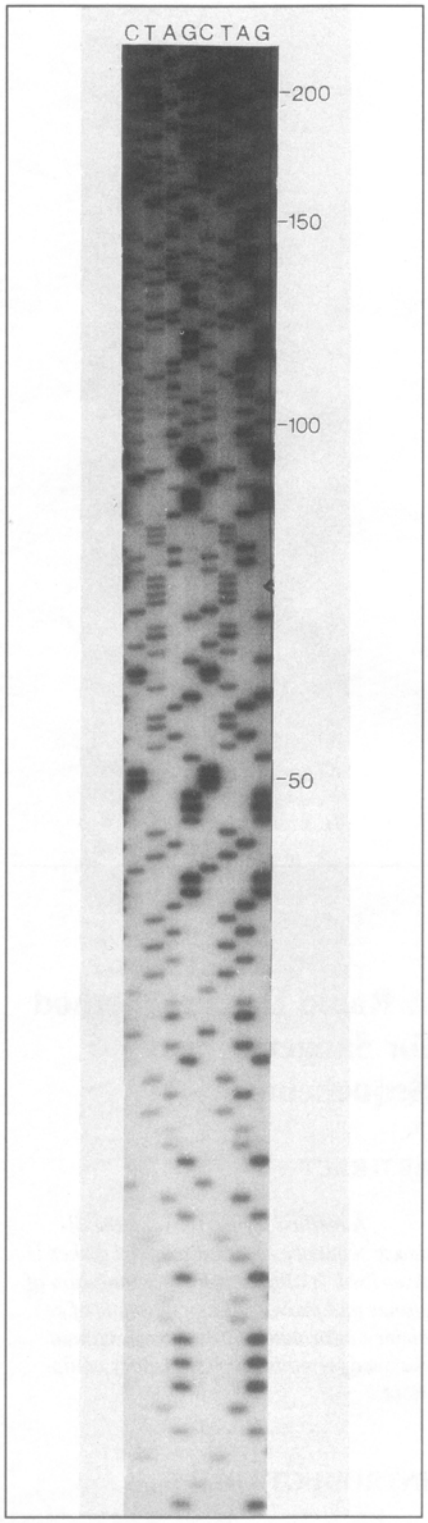

Figure 3. [α-^{32}P]dATP dideoxy sequencing of a double stranded DNA template of the cytochrome *c* peroxidase gene cloned in pEMBL8+. The samples were electrophoresed on an 8% polyacrylamide wedge shaped gel (48 lanes) at 1500 volts for 1.5 h. The overnight exposure allows 200 nucleotides to be ascertained from this short electrophoresis run. With a shorter exposure time, a further 50 nucleotides can be read.

double stranded template pEMBL DNA. Similar results have been obtained using single stranded M13 vectors and double stranded yeast shuttle vectors as DNA templates. In our hands there is no difference between the petri dish method of sequencing in comparison with the standard method. With the use of additional modifications, such as optimizing levels of ^{35}S dATP for shorter exposure time (11), two to five times more sequence data could be obtained within a day.

CONCLUSIONS

This report describes a simple and inexpensive method for speeding up DNA sequencing without the use of major pieces of laboratory equipment. A large number of clones can be manipulated at one time to yield large amounts of sequence information. This method has been effectively applied in the screening of libraries for nucleotide point substitutions (7) or for DNA sequence determination of a newly cloned gene (6). We anticipate this method to be widely used especially in combination with the rapid methods of isolation of ssDNA such as that described by Kristensen et al. (4).

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S.S. Ner, D.B. Goodin, G. J. Pielak and M. Smith
Dept. of Biochemistry and Biotechnology Laboratory
University of British Columbia
Vancouver V6T 1W5, Canada