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# DNA sequence analysis of Prinker-modified restriction fragments after collection from capillary electrophoresis with replaceable matrices

Jochen Muth[a,*], Odilo Müller[a], Jan Berka[a], Jack T. Leonard[b], Barry L. Karger[a]

[a]*Barnett Institute and Department of Chemistry, Northeastern University, 360 Huntington Avenue, Boston, MA 02115, USA*

[b]*Amicon, Inc., 72 Cherry Hill Drive, Beverly, MA 01915, USA*

**Abstract**

This paper demonstrates the procedure of sequencing DNA restriction fragments isolated by a recently developed fraction collector after CE separation. In particular, using pBr 322 plasmid as a model system, a double digest was performed with *Eco* RI and *Pst* I restriction enzymes to produce two fragments of 749 base pairs (bp) and 3612 bp, both with cohesive ends. Prinkers, specific linkers complementary to the cohesive ends, were then ligated to both fragments (increasing the size by 59 bp each). These Prinker-modified fragments were separated by CE and collected. The success of the collection was demonstrated by reinjection of each isolated fraction with laser-induced fluorescence detection, using ethidium bromide as intercalater. The 808 bp isolated fragment was then polymerase chain reaction-amplified with appropriate primers for the Prinker ends, followed by cycle sequencing. Both strands of the fragment were run on an ABI 373, sequencing 427 bases and 450 bases, respectively, with a read accuracy of 99.3%. This approach with Prinker-modified restriction fragment and automated CE fraction collection can be used as a general procedure for sequencing unknown genomic DNA as well as mutated DNA mixtures.

*Keywords:* DNA sequencing; Prinkers; DNA; Enzymes

## 1. Introduction

At present the analysis of DNA is achieved mainly by slab gel electrophoresis. An important step in the slab gel approach is the isolation of individual bands for further sample processing. However, this methodology is very time-consuming, as the gels often have to be manually prepared, and after the run, the fragments need to be cut from the gel. Subsequently, in order to isolate the DNA from the gel, the sample must be dialyzed and precipitated, both time-consuming steps that are difficult to automate.

While CE has become an important tool for the analysis of DNA [1], until recently, fraction collection has not been well developed. In the past, several approaches for collection have been offered, but these suffered from (a) significant dilution, (b) collection in a format in which the fraction was not readily available for further chemical manipulation or (c) an inability to collect multiple fractions from one run with high precision [2–5]. Automated CE systems have lately been used to collect a limited number of components exiting an open tube capillary [6–8].

Recently, our laboratory has introduced a high precision automated collection device for components exiting a column, including polymer sieving matrix filled capillaries [9,10]. The component exit

*Corresponding author.

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times from the capillary were precisely calculated from the UV trace measured with optical fibbers close to the end of the capillary. Upon the sample exiting the capillary, a sheath flow device transferred the sample components into glass capillaries without interruption of the electrical circuit. Other groups have developed sheath flow collection systems, however, without precise, on-column detection prior to collection. Furthermore, sample fraction deposition was in collection vials [11] or on epoxy coated plates [12], rather than easy-to-handle capillary tubes.

In this paper we present an integrated approach using the CE fraction collection system to isolate DNA fragments, followed by facile DNA-sequence analysis employing ligated dsDNA. In the case of mutation analysis, isolated fragments of known sequence can be readily amplified using specific primer pairs. This strategy can become complicated when many fragments must be amplified. Even more importantly, this strategy cannot be used with unknown DNA. To overcome this limitation, specific linkers were initially ligated to restriction fragments, i.e. splinkers [13], containing a self-priming stem-loop structure.

We introduce here new specific linkers, Prinkers [14], which are differentiated from Splinkers in that upon ligation to complementary cohesive ends on restriction fragments, unique priming sites for polymerase chain reaction (PCR) amplification and /or sequencing are created. The specific ligations of Prinkers to ends of the fragments also destroy the original restriction sites, thus permitting post-ligation digestion of undesirable ligation products. The priming sites carried on Prinkers are GC-rich, aiding in high stringency PCR and sequencing. These features permit direct amplification of Prinker-modified restriction fragments by PCR after CE collection, prior to sequencing.

For DNA sequencing, restriction fragments must have different cohesive ends for ligation with different Prinkers. In practice, Prinkers are ligated onto genomic restriction digests, with only some restriction fragments resulting in different Prinkers at both ends. Only these Prinker-modified fragments will be amenable to DNA sequencing. Fragments which carry the same Prinker at both ends will generate sequencing data from both ends simultaneously, making the data unreadable.

In this paper we demonstrate that Prinker-modified restriction fragments prepared by micropreparative capillary electrophoresis are suitable for PCR amplification and subsequent automated fluorescent cycle sequencing. The method which we describe is general and can be used for analysis of known fragments for mutation analysis as well as sequencing unknown DNA. In the case of known DNA, common primers can be used to amplify all Prinker-modified dsDNA.

## 2. Experimental

### 2.1. CE-Fraction Collection

For fragment isolation, a laboratory-built CE instrument was used (Fig. 1), as described previously [9]. Briefly, the collection device consisted of two parts, a detection sheath flow unit and a stepper motor controlled cylindrical holder (see inset) on which the collection capillaries were placed. The stepper motor was computer controlled and used the UV trace of the sample to align collection capillaries and the sheath flow droplet in a well-timed manner. The separation was performed in 100 $\mu$m I.D. polyvinyl alcohol coated capillaries (effective length: 13 cm, total length: 14 cm) with a high voltage power supply (Model PS/MJ30, Glassman, Whitehouse Station, NJ, USA). One end of the capillary was inserted into a 3 mL buffer reservoir containing a platinum electrode and the other end into the detection sheath flow unit. In the latter, the platinum electrode was surrounded by a sheath liquid of 40 mM Tris–N-tris(hydroxymethyl)methyl-3-

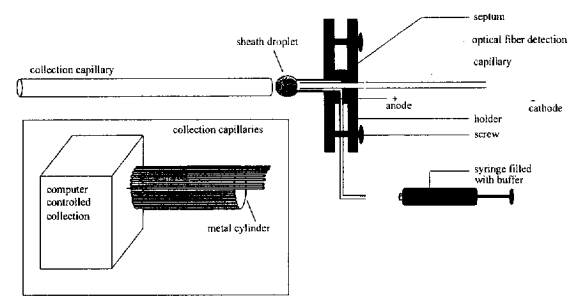


Fig. 1. Automated sheath flow CE-collection device. The samples were collected in glass capillaries. Inset: stepper motor controlled holder for collection capillaries. See text for details on the collection.

aminopropane sulfonic acid) buffer so that the fragments could be collected without interruption of the electric field (287 V/cm, $I$=14 $\mu$A). The column contained 5% (w/v) linear polyacrylamide (LPA) (Polysciences, Warrington, PA, USA), which was prepared by diluting a stock solution of 10% (w/v) LPA ($M_r \approx$700 000–1 000 000) in 40 mM Tris–TAPS buffer. The separations were performed using vacuum-degassed buffers and matrix. The polymer was electrodialyzed in the capillary for 20 min before each separation until a stable, low current was obtained. The samples were then injected for 5 s at 287 V/cm. After each run, the capillary was refilled with fresh matrix using a high pressure syringe. The fraction collector was operated with real-time data acquisition software (Labview, National Instruments, Austin, TX, USA) and a UV detector (260 nm) with optical fibbers attached 1 cm before the end of the capillary, see Ref. [9] for details. The DNA fragments were collected after peak activation and calculation of the elution time by two different methods: (a) consecutive fractions of 5 s each, starting 10 s before elution of the peak and (b) a single fraction starting 5 s before and ending 5 s after elution of the peak.

### 2.2. CE-LIF analysis

The purity of the collected fractions were analyzed on a laboratory-built laser-induced fluorescence (LIF) system. The analysis was performed in a 50 $\mu$m I.D. fused DB-capillary (effective length: 11 cm, total length: 20 cm) (J&W Folsom, CA, USA). The collected fractions were analyzed using 1% (w/v) methyl cellulose (2%=40 000 cps, Sigma, St. Louis, MO, USA) in 1×TBE buffer. The polymer matrix and buffer were vacuum degassed before each separation. The collected fragments in ~1 $\mu$L buffer were diluted in 10 $\mu$L water, injected for 5 s at 350 V/cm and reanalyzed at a field of 350 V/cm (I=11 $\mu$A). In order to detect the DNA fragments, ethidium bromide (EtBr) was added to both the separation matrix and anode buffer reservoir at a final concentration of 1 $\mu$g/mL. The DNA-EtBr complex was excited at 543 nm by a He/Ne laser (PMS Electrooptics, Boulder, CO, USA). The fluorescence emission was detected on a photomultiplier (Hamamatsu, Bridgewater, NJ, USA), after passing the light through a 543 nm blocking filter and a 610 nm band pass filter (Oriel, Stratford, CT, USA). The collected data were analyzed by real-time data acquisition software (PC NEC AT386, Turbochrom, Perkin-Elmer/Nelson, Cupertino, CA, USA).

### 2.3. Prinker-modified fragments

1 $\mu$g (0.35 pmol) of pBR322 plasmid (0.25 $\mu$g/$\mu$L, Boehringer Mannheim, Indianapolis, IN, USA) was digested with 10 units of the restriction enzymes *Eco* RI and *Pst* I (20 000 units/mL, New England Biolabs, Beverly, MA, USA). The plasmid DNA was incubated for 90 min at 37°C in the buffer supplied with the enzymes; the enzymes were then deactivated by heating the mixture at 80°C for 20 min. After inactivation, both fragments [749 base pairs (bp) and 3612 bp] of the *Eco* RI and *Pst* I digest of pBR322, which have two different cohesive ends, were ligated to their specific Prinkers.

For the ligation step, 7 pmol (0.7 $\mu$L) of the Prinkers (representing a 10-fold molar excess per template cohesive end, 10 pmol/$\mu$L) were ligated to the fragments with 10 units of T4 DNA Ligase (400 000 units/mL, New England Biolabs, Beverly, MA, USA) at 37°C for 30 min. After deactivation of the ligase at 65°C for 15 min, half of the samples were redigested with both restriction enzymes under the same conditions, as described above. A small amount of the digest ~(10 $\mu$L) was routinely analyzed by agarose gel electrophoresis using a Minisubmarine H33 (Hoefer, San Francisco, CA, USA) with 0.8% SEAKEM Gold Agarose (FMC Rockland, ME, USA), 1×TBE buffer, (ICN, Costa Mesa, CA, USA), and 1 $\mu$g/mL ethidium bromide (Sigma, St. Louis, MO, USA). After electrophoretic separation at 30 mA for 90 min, the DNA fragments were visualized on a UV-transilluminator (Hoefer). The reaction mixture containing the Prinker-modified fragments was desalted by spin column purification (CENTRI-SEP, Princeton Separations, Adelphia, NJ, USA) using a centrifuge (Model 5415 C, Eppendorf, Hamburg, Germany).

### 2.4. PCR of collected fragment

The collected short Prinker-modified fragment (808 bp) was used as template for PCR amplification. The PCR reactions were performed in a 50 $\mu$L buffer containing 40 picomoles of primer X: 5′-

CCCCCACCTCCTGCCCATCAT-3′ (20 pmol/μL, $T_m$=70°C) and primer Y: 5′-GCCCGACCCC-ACCTCCACTAC-3′ (20 pmol/μL, $T_m$=72°C), 2.5 units of *Pfu* DNA polymerase (2.5 units/μL), 5 μL 10×PCR-buffer (both Stratagene, La Jolla, CA, USA), autoclaved water (Sigma), 5 μL of 2.5 m*M* nucleotide triphosphates (Perkin-Elmer) and 2 μL of the collected DNA solution. The reactions were performed in thin wall GeneAmp reaction tubes and were covered by Ampli Wax PCR GEM 100 (Perkin-Elmer). The mixture was subjected to 25 cycles at 94°C for 45 s, 50°C for 45 s and 72°C for 2 min using a DNA thermal cycles (Perkin-Elmer). After amplification, the primers were removed by digestion with 80 units exonuclease I (10 units/μL, Amersham Lifescience, Cleveland, OH, USA) and 16 units shrimp alkaline phosphatase (2 units/μL, Amersham) at 37°C for 15 min. The enzymes were heat-inactivated at 80°C for 20 min, and the DNA was spin column-purified (CENTRI-SEP, Princeton Separations). The amount of amplified DNA was determined on an 0.8% agarose gel relative to a known DNA Mass Ladder (GIBCO BRL, Gathersburg, MD, USA).

### *2.5. Cycle sequencing of PCR amplified collected fragment*

The sequence for both strands of the fraction collected PCR amplified fragment (808 bp) was determined by cycle sequencing according to the standard method (Taq DyeDeoxy Cycle Sequencing Kit, Perkin-(Elmer). The dye reaction mixture, 4 pmol primer (primer X or Y) and 250 ng DNA as template was overlaid withAmpliWax PCR Gem 100 (Perkin-Elmer). After heating at 96°C, the mixture was subjected to 25 cycles at 96°C for 30 s, 50°C for 15 s and 60°C for 4 min. The excess of the DyeDeoxy-terminators was removed from the mixture by spin column purification (CENTRI-SEP). The desalted solution was dried in vacuum (Speed-vac-Concentrator, Savant, Farmingdale, NY, USA) and later.resuspended in 5 μL loading bufer (5 μL formamide, 1 μL 50 m*M* EDTA, pH 8). Each sample was heated at 94°C for 2 min and cooled before loading on an ABI 373 Sequencer (Perkin-Elmer).

## 3. Results and discussion

This work demonstrates the applicability of CE fraction collection of Prinker-modified DNA for sequence identification using the plasmid pBR322 (4361 bp) as a model system. To generate DNA fragments, the plasmid was digested with two restriction enzymes. a double digest of pBR322 with *Eco* RI and *Pst* I (10 units of each enzyme, see Experimental Section) produced two dsDNA fragments differing substantially in size (749 bp and 3612 bp). A series of experiments were then conducted to demonstrate that the expected products were obtained from the digestion, as described below. The results of the agarose gel electrophoretic analysis are shown in Fig. 2. Lane 1 presents the separation of the 1 kbp DNA ladder as size standard. Lane 2 represents the undigested plasmid pBR322 and lanes 3 and 4 the linearized plasmid with *Eco* RI and *Pst* I, respectively. As expected, the supercoiled form (lane 2) migrates faster than the linear form (lanes 3 and 4). Lanes 7 and 8 show the expected 749 and 3612 bp fragments obtained by digesting the plasmid with both *Eco* RI and *Pst* I.

The digestion products were next ligated to the appropriate Prinkers (Eco X and Pst Y, see Fig. 3 for sequence) using 10 units of T4 DNA ligase. The ligation products are shown in lane 5 of Fig. 2. The shorter fragment (originally 749 bp), upon ligation, increased in length by the expected 59 bp of the Prinkers (808 bp), representing a shift to longer migration time. This 59 bp shift was not detected for the larger fragment due to the limited resolving power of the gel in this higher bp region. Note that in lane 5 the excess Prinker and Prinker dimers can be seen at the bottom of the gel. Finally, a second digest of the ligated sample with 10 units of each restriction enzyme did not change the migration (lane 6), confirming that the restriction site was not reformed by the ligation of the Prinkers to each fragment end. In summary, the results in Fig. 2 demonstrate that digestion and ligation occurred, as expected.

We next employed the CE collection device for fragment isolation. For electrokinetic injection, the samples were first desalted by spin column purification followed by separation of both fragments (808 bp and 3671 bp) and isolation by the automated CE

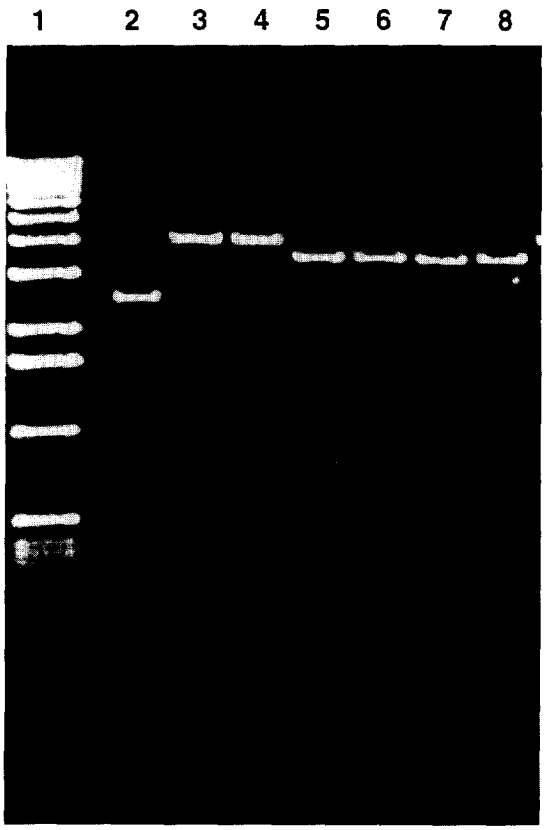


Fig. 2. Gel analysis of pBR322 after digestion with restriction enzymes *Eco* RI and/or *Pst* I and ligation with their compatible Prinkers. Lane 1:1 kbp DNA. ladder; lane 2: pBR322; lane 3: pBR322/*Eco* RI digest; lane 4: pBR322/*Pst* I digest; lane 5: Prinker-modified fragments of pBR322/*Eco* RI and *Pst* I digest; lane 6: second *Eco* RI/*Pst* I digest of sample in lane 5; lanes 7 and 8: pBR322/*Eco* RI/*Pst* I digest.

collection device. For collection, glass capillaries were mounted on the outside of a metal cylinder, see inset of Fig. 1. The metal cylinder was rotated by a stepper motor that was controlled by a PC coupled to the UV detector. The high accuracy and time resolution of this device was the result of two special features. First, the samples were detected one cm before the end of the capillary via optical fibbers (detection at 260 nm), and, secondly, a sheath flow device that allowed continuous collection without interruption of the separation run was used. The high precision and accuracy of this approach was previously demonstrated in the collection of all 11

**Eco X Prinker (*Eco* RI, *Tsp*509 I, *Apo* I, *Mun* I)**

5'-CCCCCACCTCCTgCCCATCATAAAAAATC
3'-ggggggTggAggACgggTAgTATTTTTTAgTTAAp

**Pst Y Prinker (*Pst* I, *Nsi* I, or *Sse*8387 I)**

5'-gCCCgACCCCACCTCCACTACAAAACCATTgCA
3'-CgggCTggggTggAggTgATgTTTTggTAp

Fig. 3. Sequences of two Prinkers used to prepare restriction fragments of pBR322 for PCR amplification and sequencing. The endonucleases shown in parentheses generate restriction fragments which can be ligated to each respective Prinker, although only *Eco* RI/*Pst* I restriction fragments were utilized in this study. The underlined sequences represent the priming sites for the PCR and sequencing. Primer X and Primer Y (see Experimental Section) are complementary to the respective priming sites. Terminal 5′-phosphate groups (p) are on the lower strands to allow ligation of the priming site to the terminal 3′-hydroxyl groups on compatilble restriction fragments.

fragments generated by a *Hae*III digest of $\phi$X174 [9].

In this work, both Prinker-modified fragments were separated in a short time at 287 V/cm using 5% (w/v) linear polyacrylamide as a sieving matrix (Fig. 4). After electrokinetic injection for 5 sec, the separation was performed in a short capillary and the fragments recovered in the glass capillary tubes. The peaks with a migration time shorter than 6 min, attributed to the unligated Prinkers and Prinker

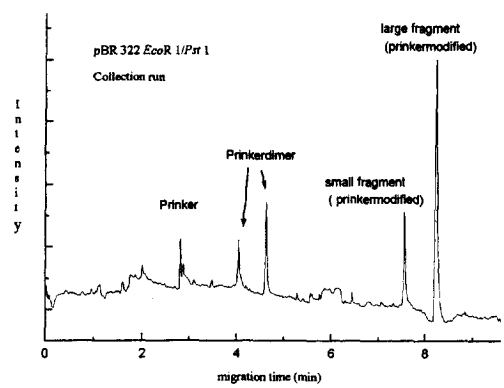


Fig. 4. Separation of Prinker-modified fragments from pBR322/*Eco* RI and *Pst* I double digest. (Conditions: $l$=13 cm, $L$=14 cm, 100 $\mu$m I.D., polyvinyl alcohol coating, 5% linear polyacrylamide, 40 mM Tris–APS buffer, 287 V/cm, 14 $\mu$A, UV detection at 260 nm. Injection: electrokinetic for 5 s at 287 V/cm. The large and small fragment were collected for subsequent analysis.

dimers, were not collected. The Prinker-modified fragments were collected by two separate methods: multiple fractions in predefined time windows (peaks collected in 5 s steps, starting 10 s before elution) or in one fraction starting 5 s before and ending 5 s after elution of the peak. After collection of the smaller fragment of digested pBR322 at 7.6 min and the larger one at 8.2 min in ~1 $\mu$L of 40 m*M* Tris–TAPS buffer, the fragments were reanalyzed for purity in 10 $\mu$L water by CE–LIF detection with 1% methyl cellulose (linear polyacrylamide can be also used) and EtBr as intercalator (Fig. 5). Using excitation at 543 nm with a He/Ne laser and fluorescence detection with a photomultiplier at 610 nm, both fragments were individually observed, indicating the success of the collection and isolation procedure. Both of the above collection protocols resulted in isolation of the two fragments.

The nucleotide sequence of the short fragment was next determined by cycle sequencing. In order to obtain sufficient material for sequencing with dye terminators (>200 ng), it was necessary to re-amplify the collected fraction. The template solution of the collected material (808 bp) was amplified by PCR using the primers X and Y described in the Experimental Section. After digestion of the excess single-stranded PCR primers and nucleotide triphosphates with exonuclease I and shrimp alkaline phosphatase as well as removal of the nucleosides and phosphates by spin column purification, the amount of amplified DNA was estimated by comparison with a known mass and size standard using slab gel electrophoresis. This estimate is necessary for the following cycle sequencing step.

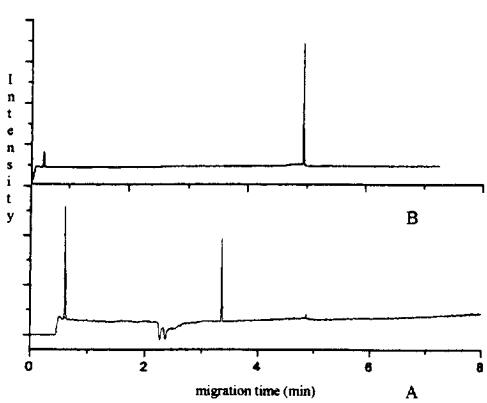


Fig. 5. Re-injection of the collected fractions and analysis by LIF detection A: 808 bp Prinker-modified fragment; B: 3671 bp Prinker-modified fragment. Conditions: l=11 cm, L=20 cm, 50 $\mu$m I.D., DB-1 coating, 1% methyl cellulose, 1×TBE buffer, 350 V/cm, 11 $\mu$A, laser ex, 543 nm. Injection: electrokinetic for 5 s at 350 V/cm.

From 1 $\mu$g PCR product, 250 ng was used as template for cycle sequencing with dye labeled terminators. The 808 bp fragment was sequenced from both ends using primer X or primer Y according to the cycle sequencing protocol for dye terminators described in the Experimental Section. The excess of DyeDeoxy Terminators was removed by spin column purification. The complete sequence of the smaller Prinker-modified fragment was obtained from both ends on a standard ABI slab gel instrument, as illustrated in Fig. 6A and B. The intensity of both reactions was high, and the sequence of the fragment was readable to roughly 450 bases from the Eco X Prinker and 427 bases from the Pst Y Prinker, with an accuracy of higher than 99.3% in both cases. It should be noted that CE could also be used for DNA sequencing [15]; however, at the time this work was performed, proper filters for these specific terminator dyes were not available for our CE unit.

## 4. Conclusions

Using pBR322 as a model system, we have demonstrated that Prinker-modified restriction fragments can be effectively purified by CE, PCR amplified, and sequenced. CE should thus be useful to prepare highly purified restriction fragments from complex digests prior to PCR amplification.

The new collection device in combination with Prinkers can be an effective approach for sequencing small amounts of material without a subcloning amplification step. It is possible that Prinkers could also be effectively combined with random sequencing approaches [16] to sequence portions of subclones from sheared cosmid DNA. One of us (JTL) has prepared a wide variety of cohesive ends on a series of Prinkers. Endonucleases which cleave different 4 bp recognition sites could be combined to generate complex digests of cosmid DNA. One of us (JTL) has prepared a wide variety of cohesive ends on a series of Prinkers. Endonculeases which cleave different 4 bp recognition sites could be combined to

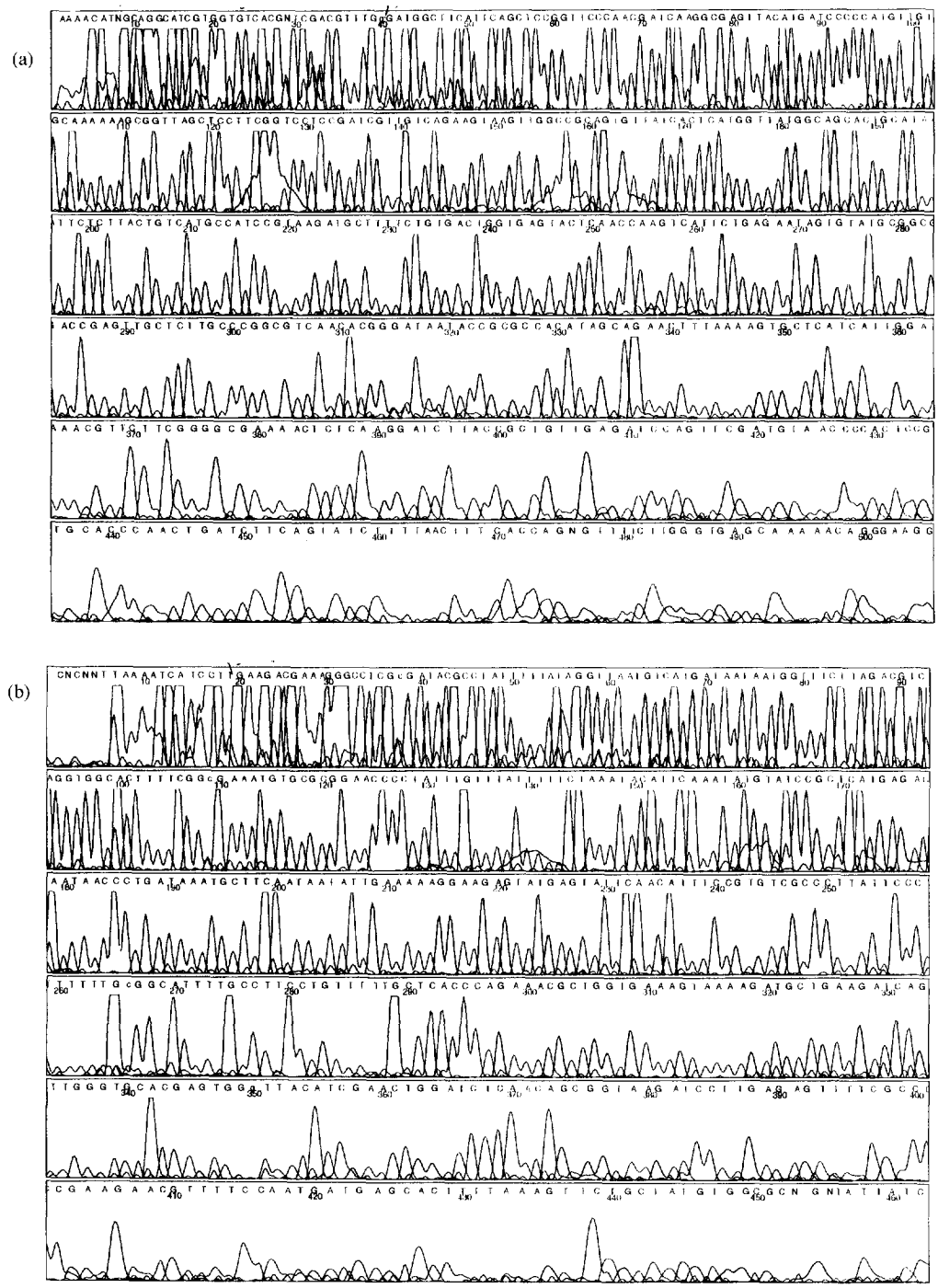


Fig. 6. Electropherogram of the ABI 373 slab gel sequencing runs for both strands of the 808 bp Prinker modified fragment (primers X and Y). The sequence data were obtained by using 250 ng of the PCR amplified fragments as template for performing the sequencing reactions, (a) Primer X. (b) Primer Y.

generate complex digests of cosmid DNA for ligation with the library of Prinkers. This would provide a variety of Prinker-modified fragments for collection, amplification, and ultimately, for the production of sequence data. Furthermore, since detailed restriction maps are available for known DNA (e.g., cDNA and diagnostic PCR products), Prinkers could be targeted to a multitude of specific restriction fragments after amplification. This would make most regions accessible to secondary PCR and DNA sequencing using a single pair of primers after CE collection.

In a new PCR-based fingerprinting technique called AFLP [17], oligonucleotide adapters were ligated to complex restriction digests prior to PCR amplification and analysis on polyacrylamide gels. The conditions described by the authors allowed selective amplification of doubly-digested restriction fragments and analysis of dozens of amplified fragments from complex restriction digests. Based on the results of this paper, the AFLP procedure could in principle be incorporated into CE with fraction collection to amplify sequencing templates from highly restricted cosmid DNA. Amplification of individual fractions would thus permit the production of predominantly random, non-redundant sequence data from cosmid DNA.

In large scale sequencing projects, the automated collection system followed by PCR and DNA sequencing demonstrated in the present paper could effectively reduce the number of random subclones required to reconstruct a continuous DNA sequence. Highly accurate and precise CE collection in combination with Prinkers is a fast, automated approach to allow subsequent manipulation in comparison with the tedious collections of fragments from the slab gel.

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