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Rapid separation, quantitation and purification of products of polymerase chain reaction by liquid chromatography

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ABSTRACT

The polymerase chain reaction (PCR), a new, powerful method for rapid enzymatic amplification of specific DNA fragments, has gained tremendous popularity in molecular biology. This paper describes the successful application of liquid chromatography to the analysis of products of the PCR. Efficient separation of both DNA restriction fragments and amplified PCR products were achieved in 10–12 min on a new ion-exchange column, DEAE-NPR, packed with 2.5-μm non-porous particles. The PCR products were quantitated with a reproducibility within 10%. Use of liquid chromatography was demonstrated for separation and quantitation of PCR products in amounts below those required for direct analysis by ethidium bromide gel electrophoresis or a Hoechst 33258 dye-based fluorescence assay. Liquid chromatography was also demonstrated to be effective for quick optimization of PCR procedures.

INTRODUCTION

The polymerase chain reaction (PCR)[1,2] is an *in vitro* enzymatic amplification of specific DNA sequences directed by two oligonucleotide primers chosen to complement opposite strands of the target sequence of a DNA molecule. After annealing the two primers to the complementary sequences of the denatured DNA, extension of the annealed primers proceeds with a thermostable DNA polymerase. This cycle is commonly repeated 20–30 times. Since the product of primer extension essentially doubled after each cycle, exponential accumulation of target DNA is expected to take place. Parameters, such as melting, annealing, and extension temperatures, primer and enzyme concentrations, and initial concentration of DNA template must be considered in achieving amplified PCR products with high yields[2]. Generally, a 10^5-fold DNA amplification can easily be generated in 3 h. Speed, specificity and automation make the PCR technology extremely attractive for use in genetic analysis and detection of infectious diseases. Following the PCR process, amplified DNA must be detected

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and characterized. However, many post-PCR detection techniques, such as ethidium bromide gel electrophoresis, are time-consuming and complicated, often requiring considerable manual dexterity, and cannot be easily automated.

During the last ten years, liquid chromatography (LC) has become a well-established analytical technique, permitting very rapid separations and sensitive detection of complex mixtures of low-molecular-weight compounds. A better understanding of solute dispersion phenomena has increased sample throughput and detection sensitivity by optimizing column length, column radius and particle size of the packing material[3]. In recent years high-performance LC (HPLC) has also become the dominant method for the purification of biopolymers, even though these separations are not as efficient or rapid as those provided by other analytical techniques, such as high-voltage gel electrophoresis. However, new stationary phases that allow much faster and more efficient separations of biomolecules have recently become available.

Two decades ago, Horváth and co-workers[4,5] developed columns packed with fluid-impermeable (non-porous) spheres, coated with ion-exchange resin, and applied them to the fast analysis of nucleic acid fragments. The authors demonstrated improved column efficiency due to the reduced pathlength of diffusion within the solid phase. The disadvantage of these originally developed non-porous particles was their greatly reduced surface area and limited sample capacity.

Recently, Anspach *et al.*[6] returned to the concept of non-porous particles, mainly to test their hypothesis that non-porous particles would improve mass recovery of proteins. They synthesized 1-μm spherical non-porous silica particles for use in affinity chromatography. Due to their small size, the surface area of the beads is only about an order of magnitude smaller than that of 10-μm macroporous particles, thereby reducing the loss of retention associated with non-porous particles, while still taking advantage of higher mass recoveries due to the absence of pores. An increase in the retentive capacity of non-porous media has also been considered by Khalghatgi and Horváth[7] who prepared micropellicular (C_8-bonded) silica particles for the fast analysis of tryptic digests. Subsequent work by them and others has demonstrated the advantages of non-porous media for the analysis of proteins and peptides in almost all modes of liquid chromatography[8,9].

Non-porous resin columns were first described by Burke *et al.*[10]. They developed 7-μm polymethacrylate beads which were subsequently marketed for separations of monoclonal antibodies. In 1987, Kato *et al.*[11] demonstrated that use of 2.5-μm non-porous ion exchangers resulted in a 5- to 10-fold decrease in protein analysis times compared to chemically equivalent 10-μm porous ion exchangers.

The above-described trend towards the use of small, non-porous particles has also benefited the separation of DNA fragments. Following their work on oligonucleotides[12], Kato *et al.*[13] recently described highly efficient and rapid separations of typical DNA restriction fragments by anion-exchange chromatography. Using a column packed with 2.5-μm diethylaminoethyl-bonded non-porous resin particles (DEAE-NPR), the authors demonstrated the separation of practically all 22 restriction fragments in a pBR322-Hae III digest in less than 15 min, and the separation of a λDNA-Hind III digest in approximately 5 min. Their results suggest that the use of non-porous resin-based anion exchangers may complement or replace the use of traditional electrophoretic techniques.

Many PCR applications require isolation of an amplified DNA segment in solution for subsequent post-PCR analyses. Since the reaction products are typically purified by gel electrophoresis, an amplified product must be recovered from a gel that requires additional experimental steps to remove contaminants introduced from the gel[14,15]. However, LC can be successfully utilized for this purpose[16].

Modern LC appears to be ideally suited for fast separation and quantitation of amplified PCR products in solution in a single step. The goal of the present work, therefore, was to explore the feasibility of using an anion-exchange column, DEAE-NPR, for rapid separation and quantitation of PCR-amplified DNA fragments. Of primary importance were the stability and maintenance of the column when the samples were chromatographed without any sample preparation prior to injection.

EXPERIMENTAL

PCR amplifications

Reagents and PCR method. Amplifications were carried out with the Perkin-Elmer Cetus GeneAmp™ PCR reagent kit (Norwalk, CT, U.S.A.). A 500-nucleotide segment of bacteriophage λDNA (nucleotides 7131 to 7630) was used as a target, the initial concentration of which was 10 pg/100 μl or $3 \cdot 10^{-13}$ *M*. Two 25-base pair oligonucleotides, PCR01 and PCR02, were employed as primers, and Perkin-Elmer Cetus native Taq DNA Polymerase or AmpliTaq™ DNA Polymerase as the thermostable enzymes. Also, Tth DNA polymerase (Finnzymes, Finland) was used as another thermostable enzyme. The standard GeneAmp PCR Reagent Kit procedure, recommended by the manufacturer, was employed for amplifications of a 500-base pair product, unless otherwise indicated: three-temperature PCR was carried out for 1 min at 94°C, 2 min at 37°C and 3 min at 72°C for 25 cycles, using the Perkin-Elmer Cetus DNA Thermal Cycler.

To amplify a 4170-base pair segment of the λDNA, the primers used were PCR01 and a 24-mer synthesized using a custom-made DNA synthesizer. The sequence of the 24-mer was as follows: 5′-AATCTGCTCCGCCGACACGTTATG-3′ (nucleotides 7131 to 11 300). Two-temperature PCR was carried out for 1 min at 94°C and for 10 min at 70°C for 25 cycles.

Analysis. A 10-μl volume of amplified 500- and 4170-base pair products were electrophoresed on a 1% agarose gel (International Biotechnologies, New Haven, CT, U.S.A.), mixed with a 3% NuSieve™ agarose (FMC, Rockland, ME, U.S.A.), and on a 1.6% agarose gel, respectively, in Tris–borate electrophoresis buffer at 10 V/cm. DNA was detected by staining with 0.1 μg/ml ethidium bromide.

pBR322-Hae III, λDNA-Hind III and λDNA-EcoR I, Hind III digests (Sigma, St. Louis, MO, U.S.A.) were used as the molecular-weight markers. pBR322 DNA, completely digested by Hae III, yields 22 fragments: 587, 540, 504, 458, 434, 267, 234, 213, 192, 184, 124, 123, 104, 89, 80, 64, 57, 51, 21, 18, 11 and 8 base pairs. λDNA digested by Hind III yields the following fragments: 23 130, 9416, 6557, 4361, 2322, 2027, 564 and 125 base pairs. A λDNA-EcoR I digest, digested by Hind III, yields 13 fragments: 21 226, 5148, 4973, 4277, 3530, 2027, 1904, 1584, 1330, 983, 831, 564 and 125 base pairs.

Yields of PCR amplifications were quantitated by a fluorescence assay, employing Hoechst 33 258 dye (Behring Diagnostics, La Jolla, CA, U.S.A.)[17].

Liquid chromatography analysis

Apparatus. The system consisted of a Perkin-Elmer Series 410 BIO pump, a Model LC-90 BIO UV detector and a Rheodyne Model 7125Ti injection valve, equipped with a 10-μl loop. To check the purity of amplified DNA samples, a Perkin-Elmer LC-235 diode-array detector was also employed. Chromatographic data were collected, using an LCI-100 laboratory integrator.

A polymer-based TSK DEAE-NPR column (Perkin-Elmer), 35 mm × 4.6 mm I.D., packed with 2.5-μm particles, was used. A scavenger C_{18} column was placed between the pump and the injector.

Reagents and methods. HPLC-grade water (Brand-Nu Labs., Meriden, CT, U.S.A.) was employed for the mobile phase. The mobile phase was prepared as follows: reservoir A contained 0.25 *M* NaCl and 20 m*M* Tris–HCl (Boehringer Mannheim, Indianapolis, IN, U.S.A.) (pH 7.7); reservoir B contained 1 *M* NaCl and 20 m*M* Tris–HCl (pH 7.7). Two gradient programs were employed: (1) the mobile phase was linearly changed from 0 to 25% B in 0.1 min, then from 25 to 30% B in 2.9 min, and from 30 to 60% B in 20 min (method 1); (2) the mobile phase was changed from 20 to 30% B in 0.1 min (concave curve with steepness of 2); then linearly from 30 to 45% B in 2.9 min, and from 45 to 50% B in 9 min (method 2). In both gradient programs, 100% B was used for clean-up before reequilibration of the column with the initial mobile-phase composition for the next injection. The column was operated at 1 ml/min at room temperature, and the LC-90 UV detector was set at 260 nm throughout the experimental work.

RESULTS AND DISCUSSION

Chromatography of PCR product

Use of a DEAE-NPR column packed with non-porous 2.5-μm particles for the separation of oligonucleotides has been described recently by Kato and co-workers[12,13]. A separation of a pBR322-Hae III digest, obtained on a DEAE-NPR column by method 1 is shown in Fig. 1A. It is seen that adequate separation of almost all DNA fragments in this sample was achieved in 22 min. It should be noted that an optimized separation and identification of the peaks were beyond the scope of this work, since parameters such as temperature, ionic strength and mass load on column efficiency have already been investigated[12]. However, some of the DNA fragments that are of interest for the separation of PCR products amplified in this work are identified in the chromatogram, following the assignment of the peaks given by Kato *et al.*[13].

One of the important aspects of the PCR is that the reaction generates relatively simple mixtures of amplified products since selective amplification of a specific sequence generally takes place. Furthermore, if the conditions of the reaction are optimized, PCR will often amplify only the targeted sequence. It follows that, depending on the nature of amplified samples, the gradient profile can be adjusted to minimize the analysis time. Method 2 was tailored to the separation of 500-base pair products from smaller size products. A separation of a pBR322-Hae III digest, obtained by method 2, is shown in Fig. 1B. It is seen that the DNA fragments were separated in less than 15 min, while a very good resolution of the peaks was still maintained. Chromatograms of 500-base pair products from two different amplifications are shown in Fig. 2. Chromatogram A demonstrates an example of a PCR

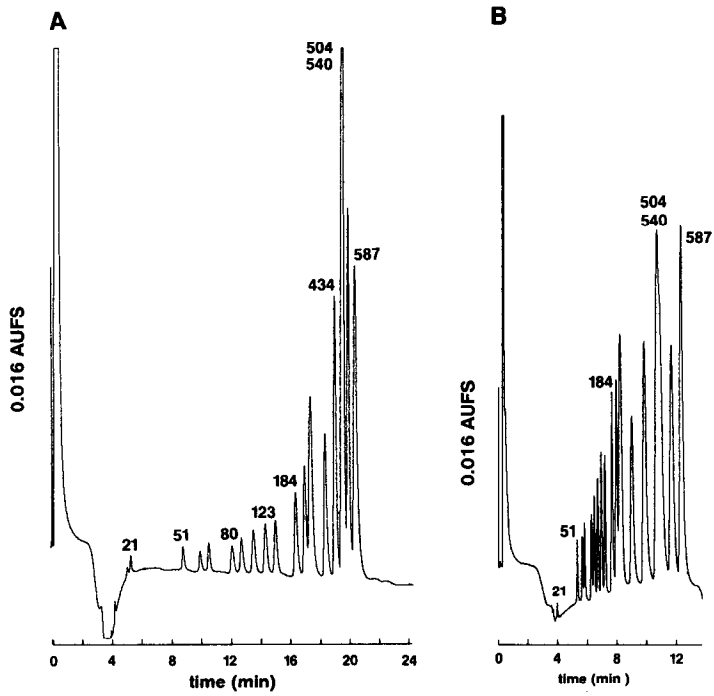

Fig. 1. (A) Separation of pBR322-Hae III digest. Sample size 20 μl of 50 μg/ml; method 1. (B) Separation of pBR322-Hae III digest. Sample size 10 μl of 50 μg/ml; method 2. Numbers at peaks indicate numbers of base pairs.

amplification where only one peak, attributed to the 500-base pair product, was generated. Chromatogram B demonstrates the separation of a PCR mixture obtained after an amplification during which a non-specific product, primer dimer, together with the 500-base pair product, was generated. In both chromatograms, identification was on the basis of the retention of appropriate DNA fragments in the pBR322-Hae III digest and confirmation by the electrophoretic separation of both the digest and 500-base pair products on 1% agarose gel.

For the PCR mixture chromatographed in Fig. 2B a very high enzyme concentration was employed, since the effect of the AmpliTaq DNA Polymerase concentration on the yield of the PCR process was studied. However, it is known that PCR amplifications in which high concentrations of primers or enzyme are employed can lead to the formation of primer dimer[18]. Also, high levels of primers and enzyme are likely to cause amplification of non-specific sequences when low concentrations of target, long annealing and extension times, and high cycle numbers are used. In these cases, simple quantitation procedures, such as a Hoechst 33 258 dye-based fluorescence assay, cannot be utilized, since they do not discriminate between responses from different DNA molecules. Therefore, a separation method must be employed to detect the amplified DNA target. Agarose gel electrophoresis can be used for the separation of amplified products, but if needed for further manipulations, these products cannot easily be recovered from the gel. In the chromatograms shown in Fig. 2A and B, the

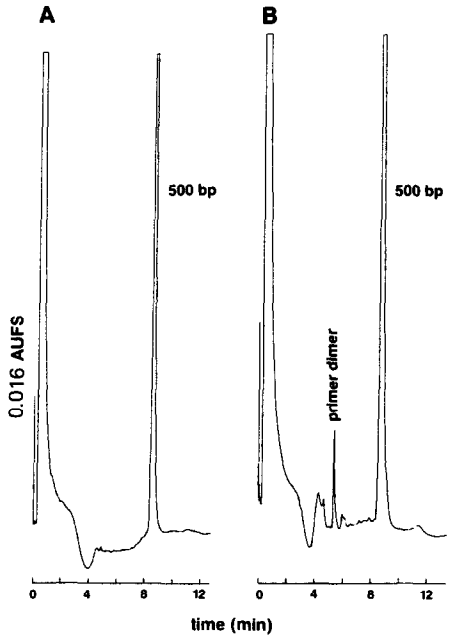

Fig. 2. Separation of 500-base pair (bp) PCR products. Sample size 10 μl; method 2, except pH 9.1 was used and the mobile phase was changed from 45 to 50% B in 7 min (step 3). PCR amplifications: three-temperature PCR. (A) Primer concentration, 0.2 μM, Taq DNA polymerase, 5 U/100 μl, 500-base pair product was amplified to 1800 ng/100 μl. (B) Primer concentration, 0.2 μM, AmpliTaq DNA polymerase, 17.5 U/100 μl, 500-base pair product was amplified to 3300 ng/100 μl.

500-base pair fragment was separated from all components of the mixture and, furthermore, the fraction corresponding to the 500-base pair product could be collected simply either manually or with a fraction collector.

For LC to be a useful technique for purification of PCR products, the chromatographic column must provide quantitative DNA recovery. The recovery of a 500-base pair product from the DEAE-NPR column was measured by injecting the undiluted 500-base pair sample in triplicate into the column. The column was then replaced with a PTFE tube, and injections of the same mixture were repeated. The total peak area of the PCR mixture eluted from the column was 57 950 (arbitrary units) *versus* 59 210; thus, 98% of the sample was recovered. It follows that not only can the amplified DNA fragments be separated and quantitated in less than 10 min, but also they can be quantitatively recovered from the column, all in a single step.

Purity of PCR product

PCR mixtures to be amplified generally contain proteins. For example, in the case of amplification of the 500-base pair target from λDNA, the samples contained Taq DNA polymerase and 0.001% gelatin. Consequently, the purity of the peak attributed to the 500-base pair product must be established. Peak confirmation and purity determination data can be conveniently obtained from diode-array UV spectra. The methods that can be used to ascertain peak purity include the determination of

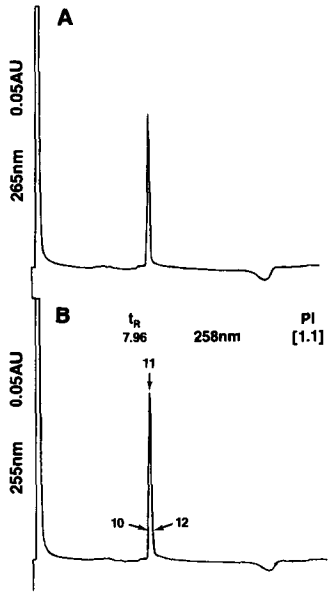

Fig. 3. Elution profiles of 500-base pair product obtained with the diode-array detector. Chromatography conditions as in Fig. 3. PCR amplification: three-temperature PCR; primer concentration, 0.1 μM; AmpliTaq DNA polymerase, 10 U/100 μl; 500-base pair product was amplified to 1900 ng/100 μl (A) 265 nm; (B) 255 nm.

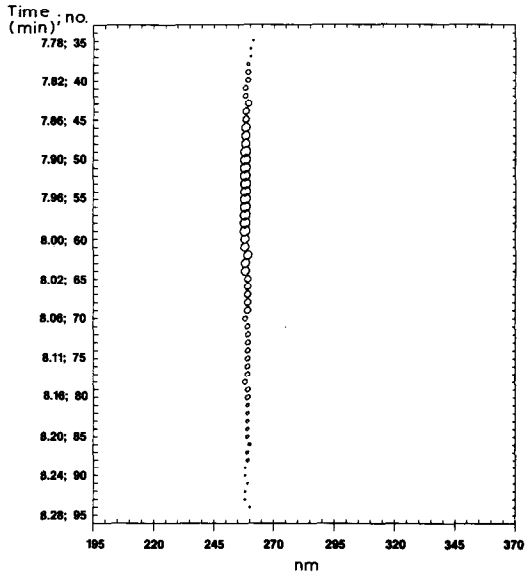

Fig. 4. Absorbance profile map of 500-base pair product. The sample is the same as in Fig. 3.

maximum absorbance wavelength, absorbance ratio plot, spectral overlay, and numerical spectral comparison (purity index). Chromatograms of a 500-base pair sample obtained with the LC-235 diode-array detector are shown in Fig. 3. It should be noted that the format of Fig. 3 is similar to the report generated by the detector software. Chromatograms A and B demonstrate the separation monitored at 265 and 255 nm, respectively. In chromatogram B, the peak eluted at 7.96 min (retention time, t_R) was scanned from 195 to 365 nm at three points, marked 10, 11 and 12. A Purity Index (*PI*) value of 1.1 was obtained by numerically comparing spectra 10 and 12 of the peak. This value, being close to 1.0, indicates that the peak was homogeneous. Also, the peak maximum at 258 nm (spectrum 11) was obtained. These results suggest that the peak represents a DNA peak not contaminated by proteins. To validate this further, an absorbance profile map can be constructed from the stored spectral data acquired during the chromatographic separation. The spectra of the same peak (t_R = 7.96 min), collected every 0.01 min, are graphically represented in Fig. 4. The collection time and spectrum number are given on the *y* axis, and the wavelength at which each absorbance maximum occurs is given on the *x* axis. The diameter of each circle corresponds to the absorbance intensity of each maximum. It is clearly seen that a straight vertical line at 258 nm was generated, and that no circles at 280 nm were observed, indicating the absence of protein contamination.

Quantitation of PCR products

It is often necessary to quantitate amplified PCR products. Using LC, a calibration curve can easily be produced for quantitation of unknown samples. Since no appropriate standards are commercially available, an LC calibration curve was generated with a 500-base pair DNA sample, amplified by the described PCR method. The specificity of the amplification was checked using the DEAE-NPR column, and

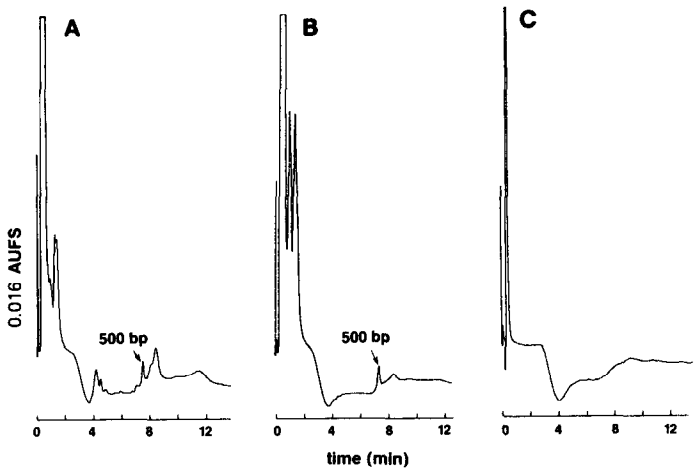

Fig. 5. Chromatograms of 500-base pair (bp) products, amplified with a thermostable enzyme. Chromatography and PCR conditions as used previously. (A) 500-Base pair product amplified to 46 ng/100 μl; primer concentration, 0.2 μ*M*; Tth DNA polymerase, 2.5 U/100 μl. (B) 500-Base pair product amplified to 40 ng/100 μl; primer concentration, 0.2 μ*M*; Tth DNA polymerase, 1.75 U/100 μl. (C) Blank chromatogram.

since the reaction yielded only one product, the Hoechst 33 258 dye-based fluorescence assay[17] was employed to determine the yield of the 500-base pair product. The DNA sample had been amplified to 2400 ng/100 μl, a typical value obtained with the standard amplification procedure and PCR conditions. The sample was then successively diluted to cover a range of concentrations from 0.24 to 24 ng/μl. A 10-μl volume of each diluted sample was injected into the column. The relative standard deviation (R.S.D.) in peak height (peak area) measurements was less than 10%. Reproducibility of the retention time was very good, the R.S.D. for 24 measurements being within 4%. The linear calibration curve with the correlation coefficient of 0.998 was obtained, which can be used for quantitation of other amplified samples.

The calibration curve was employed to quantitate PCR yields when 500-base pair products were amplified with the same standard procedure, but instead of Taq DNA polymerase, another commercially available thermostable enzyme was employed. The chromatograms are shown in Fig. 5. Distinct 500-base pair peaks were obtained in chromatograms A and B. Chromatogram C represents a blank run for comparison. Using the calibration curve, it was estimated that the yields of the 500-base pair fragments in samples A and B were 46 and 40 ng/100 μl, respectively. These values are too low for detection by the fluorescence assay and gel electrophoresis. To confirm this, both methods were applied. The fluorescence response of these products was commensurate with that of blank samples, and no visible bands on ethidium bromide-stained agarose gel could be observed.

Reproducible column performance is one of the requirements for carrying out quantitative analysis by chromatographic techniques. The small particles with which highly efficient LC columns are packed induce high inlet pressures. Therefore, some care must be taken to maintain their performance. Simple steps, such as using high-quality grade reagents, filtering mobile phases, and flushing columns with appropriate solvents, are generally recommended. In our work, HPLC-grade water and a scavenger C_{18} column were employed. The analytical column was regularly flushed with 2–3 ml of 0.2 *M* NaOH to prevent possible contamination by impurities in the mobile phase and the injected samples. These precautions allowed the retention times to be reproducible and the column inlet pressure to be kept at *ca.* 2000 p.s.i. with a flow-rate of 1 ml/min.

Although the peak area reproducibility was quite acceptable, the background level was found to increase with time, indicating accumulation of poorly eluted compounds. This could be due to the presence of large proteins in matrices of the PCR samples. It should be noted that this phenomenon did not interfere with the measurements carried out in the present work, but for very high column stability, some precautions, such as use of an appropriate guard column or organic solvent mixtures (*e.g.*, 20% aq. acetonitrile) for cleaning the column should be considered.

Optimization of PCR amplifications

One of the attractive features of the use of LC in post-PCR analysis is rapid evaluation of reaction products during tedious optimization procedures. PCR is now employed in a large number of applications in molecular biology. General guidelines for a typical reaction that can serve as a starting procedure have recently been outlined by Saiki[19] and Williams[20]. But each specific application will require that a number of different parameters affecting the PCR process be examined and controlled. Gel

electrophoresis is widely used to validate the specificity of amplifications. Fig. 6 exemplifies the electrophoresis of a targeted 4170-base pair product, amplified as described in the Experimental section, and a λDNA-Hind III digest, obtained on a 1.6% agarose gel. Electropherograms of a 500-base pair fragment and a pBR322-Hae III sample, used as control samples, are given for comparison. An additional band is seen in lane 5, corresponding to an approximately 1100-base pair product. This product was identified on the basis of the electrophoretic separation of the λDNA-EcoR I, Hind III fragments (data not shown). By changing the conditions of

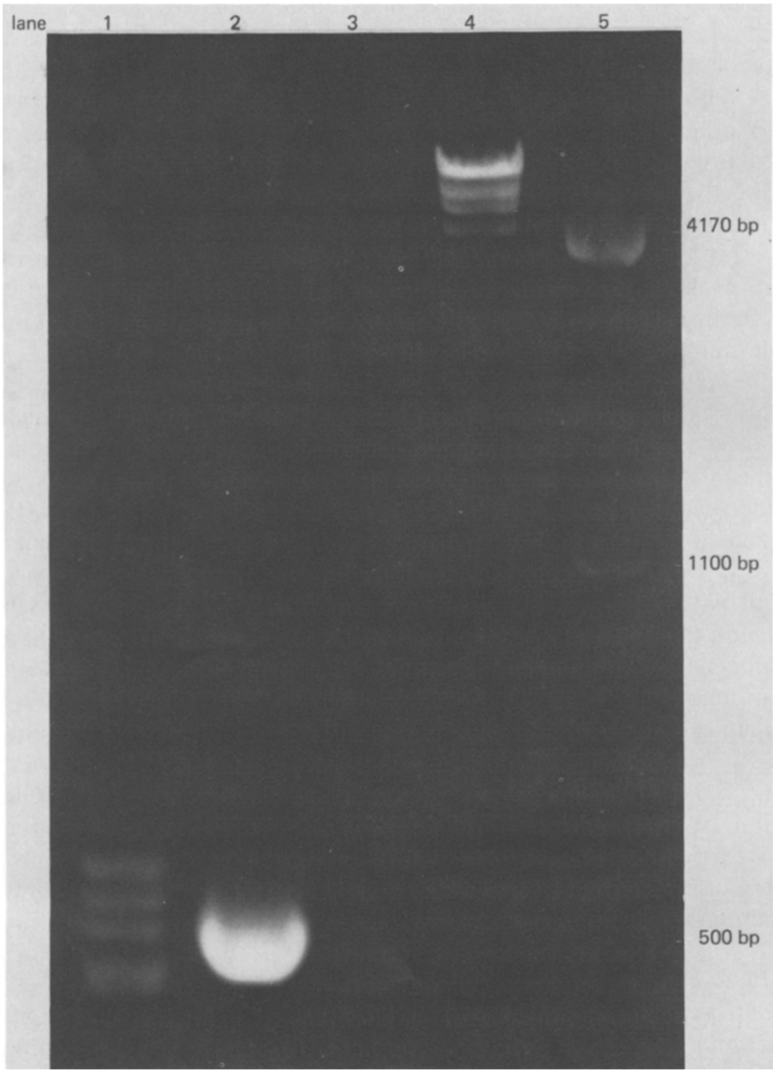

Fig. 6. Electropherogram of 4170-base pair (bp) product. PCR conditions: two-temperature; primer concentration, 0.2 μM; AmpliTaq DNA polymerase, 2.5 U/10 μl. Lanes: 1 = pBR322-Hae III (5 μl of 50 μg/ml); 2 = 500-base pair product (10 μl of 1900 ng/100 μl); 3 = λDNA-Hind III (5 μl of 50 μg/ml); 4 = 4170-base pair product (10 μl).

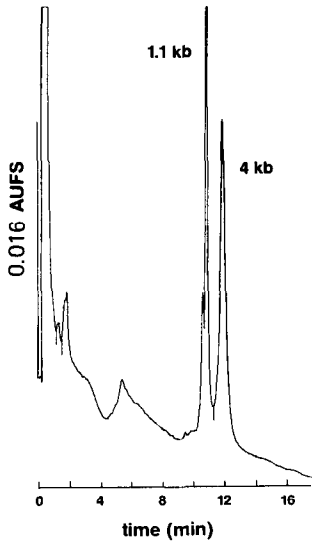

Fig. 7. A chromatogram of 4170-base pair product. Sample size 10 μl; chromatographic conditions as in Fig. 2, except pH 9.1 was used and the mobile phase was changed from 45 to 100% B in 20 min (step 3). kb = Kilobase pairs.

the PCR, the yield of this non-specific product was either enhanced or suppressed. Using 95°C for the denaturation step and 67°C for both the annealing and extension steps, only the 4170-base pair fragment was generated.

Electrophoresis of a single PCR product generally takes 1 or 2 h. Obviously, the capability of gel electrophoresis to process 16–24 samples simultaneously is not attractive during the optimization process when one parameter at a time must be changed. LC can be more efficient technique for this purpose. Fig. 7 shows a chromatographic separation of the same 4170-base pair product on the DEAE-NPR column by a modified method 2. It is clearly seen that the elution of the sample was achieved in less than 15 min, with both the 1100- and 4170-base pair fragments well separated. It follows that the next step of the optimization procedure can be initiated in a matter of minutes, not hours. Furthermore, the reaction can be monitored as it proceeds by injecting aliquots of a PCR sample during its amplification.

CONCLUSIONS

Using a DEAE-NPR anion-exchange column, the separation and quantitation of mixtures of amplified DNA fragments up to at least 4000 base pairs can be achieved in less than 15 min. At the same time, pure DNA product can be isolated from the column with quantitative recovery. Although PCR samples were injected without any sample preparation prior to injection, both the retention time and peak height (area) were reproducible, provided the column was regularly cleaned with 0.2 *M* NaOH. It follows that the LC method described can be employed successfully for rapidly separating, quantitating and purifying products of the PCR in a single run. Furthermore, the method allows much faster optimization of PCR amplifications by

replacing the gel electrophoresis method currently employed to monitor the conditions of the reaction.

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