Research Report

# Rapid Analysis and Purification of Polymerase Chain Reaction Products by High-Performance Liquid Chromatography

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**ABSTRACT**

*This report describes the use of high-performance liquid chromatography (HPLC) for the rapid analysis and purification of the polymerase chain reaction products. Employing a new anion-exchange nonporous column, efficient separations of both DNA restriction fragments and amplified PCR products are achieved in 10 to 20 minutes and quantitated within ± 10%. The performance of the HPLC technique is described in terms of resolution, reproducibility, sensitivity and micropreparative capability and compared to that of gel electrophoresis for this application.*

## INTRODUCTION

The polymerase chain reaction (PCR) is an *in vitro* enzymatic amplification of genomic or cloned target sequences directed by a pair of oligonucleotide primers (15–16). Using PCR, a $10^5$-fold DNA amplification can easily be achieved in 3 h. This powerful method has gained rapid acceptance in molecular biology. The PCR technology is currently applied in direct sequencing, genomic cloning, DNA typing, detection of infectious microorganisms, site-directed mutagenesis, prenatal genetic disease research and analysis of allelic variations (15,16,20).

Following the PCR process, amplified DNA products are usually analyzed by agarose or polyacrylamide gel electrophoresis, Southern blotting/probe hybridization or fluorescence assay (Hoechst 33258 dye) (8). Gel electrophoresis is the most common technique to separate DNA fragments owing to its high resolution (1). However, the technique is time-consuming and only semiquantitative. Also, the recovery of purified components from the gel is laborious (17,18). Probe filter hybridization is often used for the detection of a specific sequence of PCR products but suffers from the same disadvantages as gel electrophoresis (17,18). Probe hybridization in solution can be performed quickly and reproducibly, but a particular sequence of target DNA has to be known to develop an appropriate probe. The Hoechst dye assay provides quantitation, but is otherwise nonspecific (8). Thus, none of these techniques is entirely satisfactory for post-PCR analysis.

During the last decade, high-performance liquid chromatography (HPLC) has become the premier method for the analysis and purification of biomolecules (3,5). Recently, new columns packed with small nonporous particles have been developed (9,12). Not only do these columns provide fast and efficient separations of large biopolymers due to increased mass transfer between the stationary phase and the mobile phase, but also nonporous particles greatly improve mass recovery for biomolecules. Efficient separations of polynucleotides up to 20 000 base pairs (bp) have been reported previously on a newly developed nonporous anion exchanger (14). Quantitative recovery of purified fragments has also been demonstrated (13). This column advancement makes HPLC a viable alternative to gel electrophoresis for the analysis and purification of PCR-amplified DNA fragments (11).

The performance of an HPLC technique based on a new nonporous anion-exchange column is described in this paper. Performance parameters such as resolution, speed, precision, sensitivity and micropreparative capability are documented and compared to those of gel electrophoresis.

## MATERIALS AND METHODS

### HPLC System

The HPLC system used in this study consisted of the Model 250 binary BIO LC pump, the Model 7125 BIO injector and the LC-95 biocompatible UV-VIS detector. An OMEGA-2 Workstation served as the data-handling device. All instruments were from Perkin-Elmer (Norwalk, CT). The performance and specifications of the HPLC system components have been reported previously (2,7,11). The analytical column was a P-E TSK DEAE-NPR column (35- × 4.6-mm i.d.) packed with 2.5-μm particles of hydrophilic resin, bonded with DEAE groups. The ion-exchange capacities are ca. 0.15 meq/ml and the $pK_a$ values of the ionic groups are around 11.2 (12).

### PCR Amplifications

Amplifications were carried out using the Perkin-Elmer Cetus GeneAmp™ PCR Reagent Kit (Norwalk, CT). A 500-bp nucleotide segment of bacteriophage λ DNA (nucleotides 7131–7630) was used as a target, the initial concentration of which was 10 pg/100 μl or $3 \times 10^{-13}$ M. Two 25-residue long oligonucleotides, PCR01 and PCR02, were employed as primers, and Perkin-Elmer Cetus native *Taq* DNA polymerase or AmpliTaq™ DNA polymerase were used as the thermostable enzymes (15,20). The standard GeneAmp PCR Reagent Kit protocol was employed for amplification of the 500-bp product. Unless otherwise indicated, a three-temperature step cycle 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.

### Electrophoretic Analysis

Ten microliters of an amplified 500-bp product were electrophoresed on 1% agarose gel (IBI, New Haven, CT) mixed with a 3% NuSieve™ agarose (FMC, Rockland, ME) in Tris-borate electrophoresis buffer at 10 V/cm. DNA was detected by staining with 0.1 μg/ml ethidium bromide (EtdBr).

The pBR322 DNA-*Hae*III digest (Boehringer Mannheim, Indianapolis, IN) was used as the molecular weight marker. The 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 7 bp.

### HPLC Operating Conditions

The mobile phase was as follows: reservoir A contained 1 M NaCl and 25 mM Tris-HCl, pH 9.0 (Boehringer Mannheim); reservoir B contained 25 mM Tris-HCl, pH 9.0. Eluents were filtered through a 0.45-μm membrane before use. Two gradient programs were employed:

**Program #1:** 25%–45% A in 0.5 min, 45%–50% A in 4.5 min, 50%–62% A in 15 min, 62%–100% A in 1 min, 100% A for 3 min and then 100%–25% A in 1 min. All gradient segments are linear unless otherwise stated.

**Program #2**: 30%–40% A in 0.1 min (curve 2), 40%–52% A in 2.9 min, 52%–60% A in 7 min, 60%–100% A in 0.5 min, 100% A for 1 min and then 100% A–30% in 0.2 min.

Note that in both gradient programs, 100% A was used as a column cleanup step before the reequilibration of the column for 10 min with the initial mobile phase composition for the next injection. The total injection-to-injection cycle times were 35 min and 22 min for gradient programs #1 and #2, respectively.

The column was operated at 1 ml/min at room temperature, and the UV detector was set at 260 nm throughout the experimental work. The inlet column pressure for the new column was about 1000 psi at 1 ml/min. After 5–10 injections of the DNA standard, a gradual increase of column back pressure was observed until the pressure stabilized at the 1500- to 2000-psi range.

## RESULTS

### Resolution

To illustrate the resolution of the HPLC system, a separation of the restriction fragments from a pBR322 DNA-*Hae*III digest is shown in Figure 1. Gradient program #1 was used to chromatograph the sample. Excellent separation of almost all DNA fragments with near baseline resolution was achieved in 14 min. Peak elution is primarily in the order of increasing chain length, although DNA fragments having high A-T contents are often eluted later than expected (13). Assignment of peak identity was based on a similar ion-exchange separation of the same sample reported previously (14). It should be noted that digestion products from DNA *Hae*III are double-stranded blunt-ended fragments which always terminate with GG and CC. According to the chromatogram (Figure 1), it appears that small DNA fragments less than 600 bp can be separated almost completely when they differ in chain length by 5%–10%.

Resolution in HPLC is primarily controlled by column efficiency (particle size, column length), mobile phase (pH, ionic strength) and operating conditions (gradient time, flow rate) (3). By changing the gradient steepness, separation of DNA fragments from 1–20 000 bp can be optimized on this column (14). While this type of separation is also possible on conventional porous anion exchangers (although much slower), large DNA fragments often get entangled within the pore structure of the resins causing poor peak shape and lower recovery. The elimination of pores in this 2.5-μm nonporous resin is deemed responsible for excellent peak shape and short analysis time (9,13). Resolution can be further enhanced (mostly for smaller fragments) by connecting two columns in tandem (14). However, analysis time is also lengthened proportionally.

Figure 2 shows an electropherogram of the pBR322-*Hae*III digest and the 500-bp PCR products from different amplifications. It is clearly seen that the resolution achieved on the HPLC system (Figure 1) is comparable to that in the electropherogram (Figure 2, lane 1), even though the electrophoretic process takes about two hours. The gel separation of the PCR products (lanes 3–7) will be discussed later.

### Speed of Analysis

The HPLC analysis time can be further reduced by adjusting the solvent program according to program #2. As shown in Figure 3, separation time of the same digest was reduced to less than 10 min. While loss in peak resolu-

tion is evident, the separation achieved in this fast assay should still be adequate for most post-PCR analysis.

Chromatograms of the 500-bp products from two different amplifications are shown in Figure 4. Generally, the PCR process produces simple mixtures of amplified products. Under optimized conditions, PCR will generally only amplify the targeted sequence. Figure 4A demonstrates an example of a PCR amplification where only one peak, attributed to the 500-bp product, was generated (in addition to the unincorporated primers).

Figure 4B demonstrates a separation of a PCR mixture amplified using a very high enzyme concentration which led to the formation of a nonspecific product, primer dimer (19). Nonspecific sequences can be amplified when high levels of primers and enzyme are employed (19,20). This may also occur if low concentrations of target, long annealing and extension times and high cycle numbers have to be used. In such cases, simple quantitation procedures such as a Hoechst 33258 dye-based fluorescence assay cannot be utilized since they do not discriminate between responses from different DNA molecules. Agarose gel electrophoresis can be used for the separation of amplified products as demonstrated in Figure 2 (lanes 6-7), but if needed for further manipulations, these products cannot be easily recovered from the gel (17,18). In contrast, HPLC allows easy collection of the purified fragments at the detector exit tube either manually or by using a fraction collector.

The speed of the HPLC analysis allows for the rapid evaluation of reaction products that is needed during PCR optimizations. Since specific PCR applications often require adjustments of time/temperature profiles on the DNA Thermal Cycler for efficient amplification of specific target (20), the optimization process can be tedious. While gel electrophoresis is widely used to validate the specificity of amplifications, for optimization purposes, the gel preparation and development time is prohibitively long. In this case, the batch-processing advantage of gel electrophoresis to run 16–24 samples simultaneously cannot be realized during the optimization process when only one parameter at a time is changed. In addition, because gel electrophoresis is at best semiquantitative, modest differences in amplification efficiency under different conditions may not be noticed. Since many different parameters are varied in a PCR, it is important to detect even small differences because the cumulative effect of optimizing several different parameters may be large. Using the HPLC conditions shown in Figure 4, the result of each amplification can be obtained in about 10 min. Furthermore, the progress of the reaction can be monitored at each cycle as it proceeds by injecting aliquots of a PCR sample during its amplification.

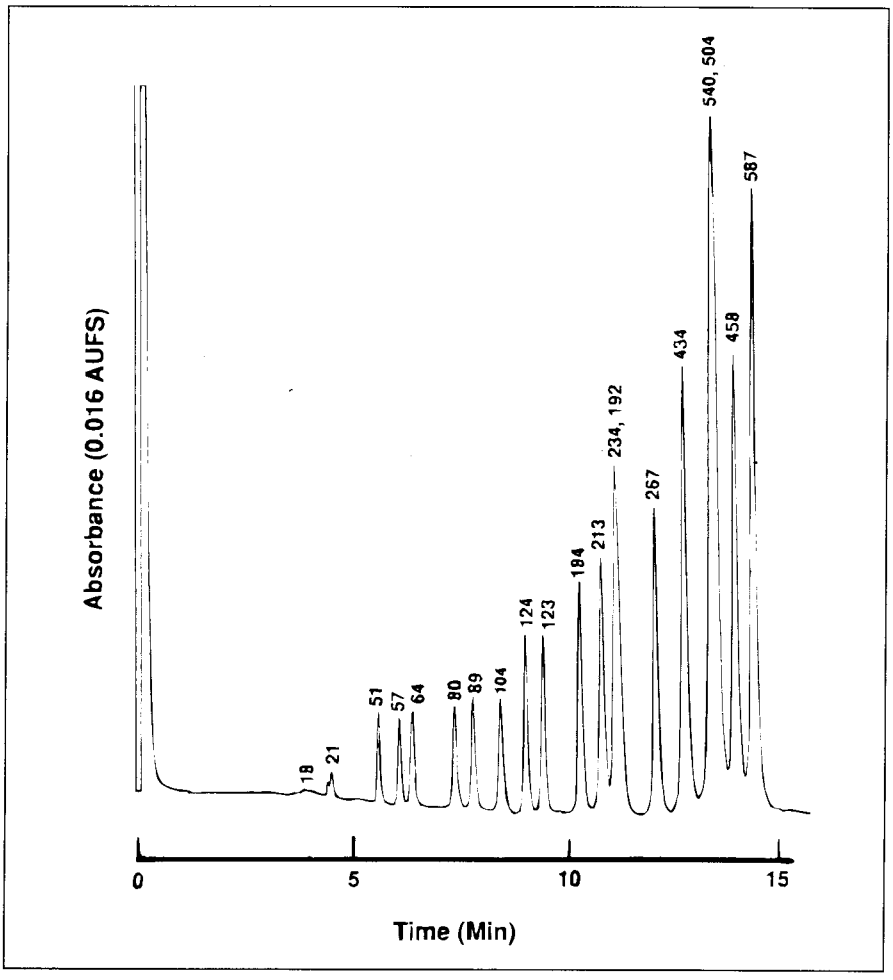


**Figure 1. Separation of pBR322 DNA-*Hae*III digest illustrating the resolution and speed of the column for oligonucleotide analysis.** Sample size: 20 µl of 50 µg/ml. Gradient program #1 was used. Size in base pairs is indicated above each peak. Absorbance scale is given in absorbance units full scale (AUFS).

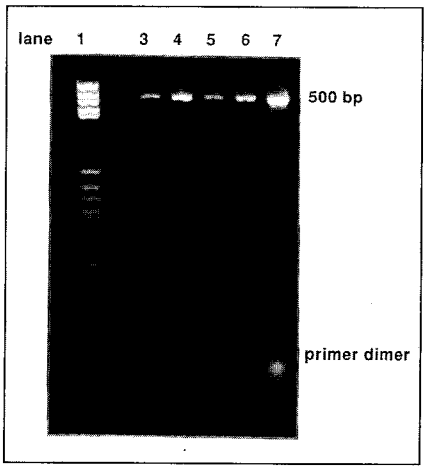


**Figure 2. Electropherogram of pBR232 DNA-*Hae*III digest (lane 1) and the PCR products produced from two separate amplifications (lanes 3–7).** See text for electrophoretic conditions and details on the samples.

## Quantitative Aspects

**Precision.** In the last decade, HPLC has evolved into a mature quantitative technique with dramatic improvements in instrumentation (10). Retention time and peak area precision in HPLC are important for both peak identification and quantitation (6).

In a previous report on peptide mapping using a similar HPLC system with an autosampler, precision levels of 0.1%–0.25% relative standard deviation (RSD) for retention time and <0.5% RSD for peak area were demonstrated (2). In comparison, by making repetitive 10-µl injections of a 500-bp product directly from the amplification tube using the manual injector, precision levels of 4% RSD (retention) and 10% RSD (peak area) were obtained using gradient program #2. While these precision values fell short of the capability of the automated peptide mapping HPLC system (2), they are significantly better than those from gel electrophoresis (1,17,18) and should be adequate for most analyses of PCR products.

**Recovery.** Quantitative recovery of a 500-bp amplification product was demonstrated by comparing the peak areas resulting from identical injections into the HPLC system: first, with the column intact and, second, with the column replaced by a Teflon® tube. Results showed that 98% of 500-bp product was recovered from the column with negligible irreversible adsorption effects. Other studies have also reported recoveries of DNA fragments ranging from 85%–100% from the same column (11,13,14). Fast recovery of a pure fragment is one of the significant advantages of HPLC. After desalting, the pure product can be used for further manipulations such as cloning, sequencing, reamplifying and labeling.

**Linearity.** Figure 5 shows a calibration curve plotting detector absorbance against the mass of injected 500-bp polynucleotide. Linear response was demonstrated from 2.4–240 ng for this sample with a coefficient of linear correlation of >0.998.

**Sensitivity.** Figure 6 shows chromatograms obtained by injecting 2 ng of the 500-bp product and a blank sample. The detection limit was estimated to be 0.3 ng at a signal-to-noise ratio of 3. This limit is significantly better than that for EtdBr gel electrophoresis (with detection limits by eye of 1–5 ng) (1). The HPLC detection sensitivity per unit mass should be

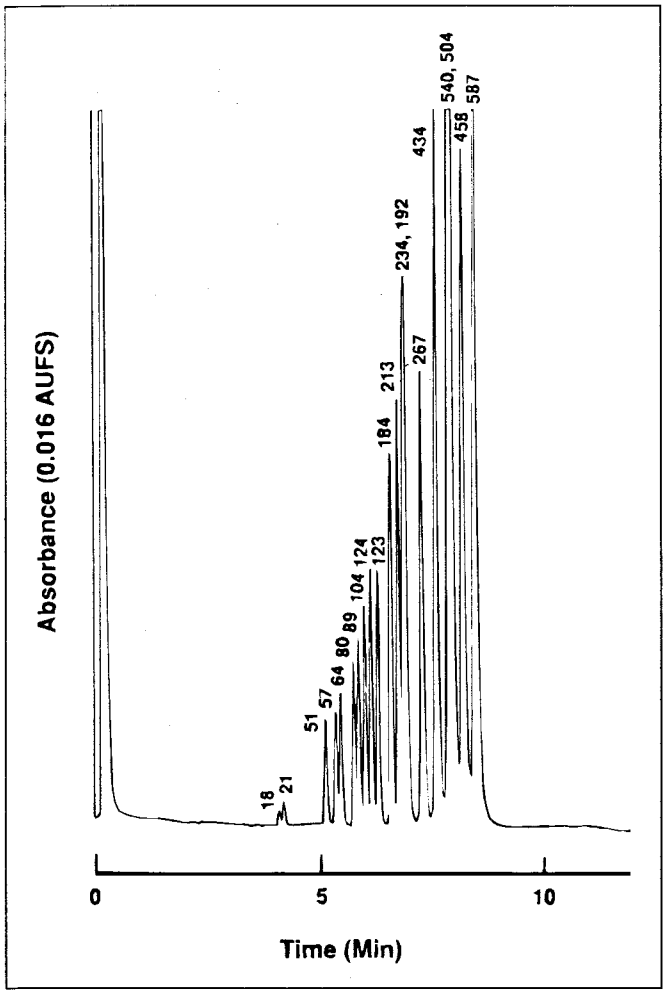


**Figure 3. Rapid separation of pBR322 DNA-*Hae*III digest.** Sample size: 10 µl of 50 µg/ml. Gradient program #2 was used.

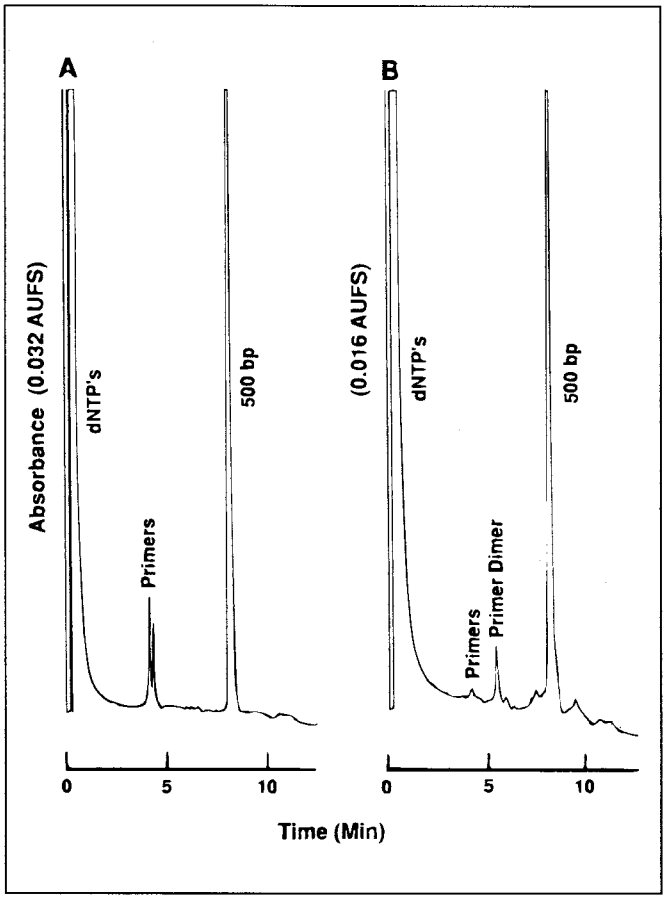


**Figure 4. HPLC separation of the 500-bp PCR products produced from two amplification experiments.** Gradient program #2 was used. Chromatogram A: primer concentration – 0.2 µM; *Taq* DNA polymerase – 5 U/100 µl; 500-bp product was amplified to 1800 ng/100 µl. Chromatogram B: primer concentration – 0.2 µM; AmpliTaq DNA polymerase – 17.5 U/100 µl; 500-bp product was amplified to 3300 ng/100 µl.

similar for single- or double-stranded DNA, a distinct advantage compared to EtdBr staining where a dramatic difference in sensitivity is observed between single- and double-stranded DNA.

### Sample Loading Capacity

The sample capacity of the nonporous column was found to be 0.5–2 µg for single components and about 5–10 µg for mixtures (14), depending on the resolution of the particular component in the sample. The loading capacity of the nonporous resins, with very low surface area, is typically 5–10 times lower than that of the comparable porous materials (13). This capacity range is adequate for isolating PCR products from most samples since the maximum amount of DNA produced in a typical 100-µl reaction volume is 2–3 µg (20).

## DISCUSSION

### Operating Conditions and Precautions

Table 1 summarizes the operating range and conditions for the nonporous DEAE column. Chromatographic selectivity can be optimized by using different mobile phases (14). Resolution appears to be optimum at about 1 ml/min (14). A longer gradient time is favored for high resolution and the gradient steepness should be adjusted to optimize resolution within any particular fragment size. Detailed discussion on the effect of operating conditions on resolution can be found elsewhere (13).

The use of highly efficient columns packed with very small particles (2.5 µm) requires extra operating care to maintain their performance. Simple steps such as using high quality grade reagents, filtering mobile phases through 0.45-µm membrane filters and flushing columns periodically with appropriate solvents are recommended.

To prevent sample carry-over, a "blank" chromatogram should be obtained before a new type of DNA sample is injected. It is especially important when the HPLC system is employed to purify PCR products since even a very low level of contamination of DNA molecules from previous runs may produce spurious results in further manipulations of purified PCR samples.

**Table 1. Operating Conditions for the P-E TSK DEAE NPR Column**

| Parameter | Typical Operating Range (14) |
|---|---|
| Mobile Phase | 0.1–1 M NaCl or 0-0.4 M $NaClO_4$ |
| | pH 7–10 |
| | Buffer 20–30 mM Tris or 1,3-diaminopropane-HCl |
| Flow | 0.3–1.5 ml/min |
| Gradient | |
| Time | 5–30 min |
| Steepness | 10–20 mM NaCl/min for <1000 bp |
| | 20–30 mM NaCl/min for 1000–5000 bp |
| | 30–50 mM NaCl/min for 5000-20 000 bp |
| Temperature | 25°–65°C |
| Pressure | 500–3000 psi |

The analytical column should be flushed with 2-3 ml of 0.2 N NaOH periodically after use (loaded from the injector) to eliminate extraneous materials adsorbed on the column inlet. The column can also be back-flushed with the mobile phase. In our laboratory, these precautions allowed us to maintain column performance and the normal back pressure range (1500–2000 psi) for at least several hundred injections. The actual column life, however, is highly dependent on the nature and amount of samples injected. The use of guard columns or in-line filters, and more effective column cleaning procedures is currently being investigated.

### Comparison with Gel Electrophoresis

Table 2 shows a comparison of HPLC performance criteria using nonporous anion exchangers with those of gel electrophoresis.

As indicated from the comparison, gel electrophoresis excels in resolution, applicability to wide molecular range and low equipment cost, while HPLC has better performance in quantitation, analysis speed, sensitivity and product recovery.

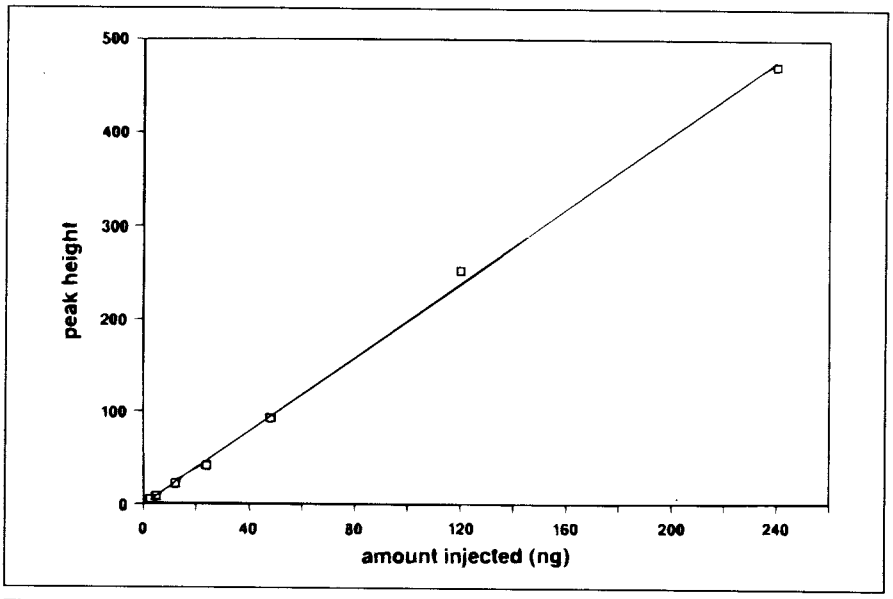


**Figure 5. Calibration curve showing absorbance at 260 nm vs. amount of the 500-bp PCR product injected from 2.4–240 ng.** Amplification conditions were the same as in Figure 4A.

Table 2. Comparative Performance of HPLC vs. Gel Electrophoresis

| Performance Criterion | HPLC | Gel Electrophoresis |
|---|---|---|
| Resolution | good | excellent |
| Molecular size range | 1–20 000 bp | 20–1 000 000 bp |
| Sensitivity | 0.2–0.4 ng | 1–5 ng |
| Quantitation | excellent ± 10% | semiquantitative ± 50%–200% |
| Preparative | | |
| Loading capacity | 0.5–10 µg | 1 µg/well |
| Recovery | 85%–100% | variable |
| Productivity | | |
| Analysis time | 10–20 min | 1–2 h/16 samples |
| Simultaneous processing | no | 16–36 |
| Automation | yes | no |
| Equipment cost | >$15 000 | $1000–$5000 |

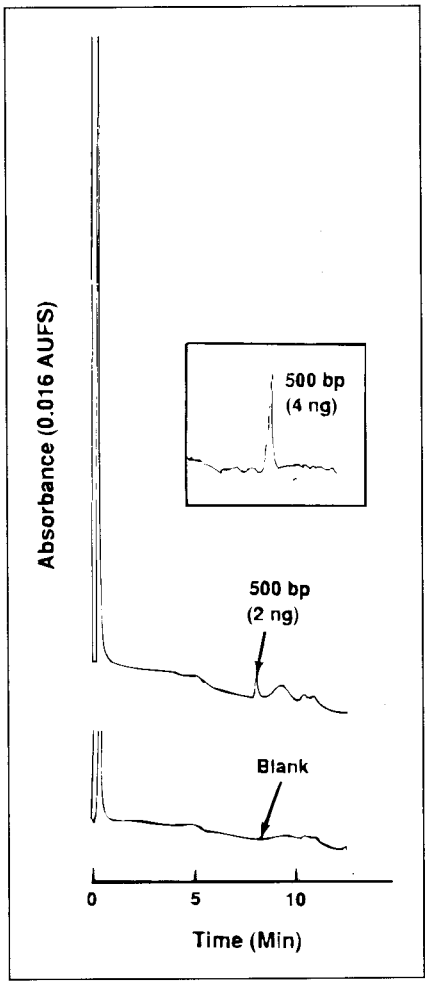


**Figure 6. High sensitivity HPLC separation of PCR products.** Chromatograms showing the blank and a sample containing 2 ng of the 500-bp product. Amplification conditions were the same as in Figure 4A. A magnified portion of another chromatogram from a 4-ng injection is shown here to illustrate the noise level. HPLC conditions used were identical to those in Figure 3.

## CONCLUSION

It has been demonstrated that the HPLC technique described can provide rapid separation, quantitation and purification of PCR products in a single run. Purified DNA samples can then be used for further manipulations such as cloning, sequencing and labeling. The HPLC technique, unlike gel electrophoresis, can also yield quantitative recovery of purified PCR-amplified DNA fragments. Due to its analysis speed, HPLC can be efficiently employed for the fast optimization of PCR process. While the equipment cost of HPLC is relatively high, the same biocompatible liquid chromatograph can be used in the molecular biology laboratory for the preparation of labeled and unlabeled primers and probes used in PCR amplification and the analysis of other biomolecules such as proteins and peptides.

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