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Conductivity detection of polymerase chain reaction products separated by micro-reversed-phase liquid chromatography

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Abstract

In this paper, we describe the application of micro-reversed-phase high-performance liquid chromatography (μ-RP-HPLC) for the separation and/or purification of polymerase chain reaction (PCR) products with detection accomplished using a miniaturized conductivity detector. The conductivity detector used two Pt wires and a bipolar waveform applied to the electrode pair from which the conductivity of the bulk solution could be measured. In the mobile phase used for the μ-RP-HPLC separation of the PCR product, the mass detection limit for herring sperm DNA using conductivity was found to be 11 ng. Efficient separation of the PCR amplicon from the other reagents present in the PCR cocktail was achieved in less than 4 min with a capacity factor of 2.5 and separation efficiency of $9.1 \cdot 10^3$ plates. The separation was carried out using reversed-phase ion-pair chromatography with a triethylammonium acetate ion-pairing agent.

Keywords: Conductivity detection; Detection, LC; Instrumentation; DNA

1. Introduction

The polymerase chain reaction (PCR) has become a powerful tool for the rapid enzymatic amplification of specific DNA fragments. The PCR can amplify genomic DNA exponentially using temperature cycles consisting of denaturation, annealing and extension and can provide sufficient amounts of material for subsequent analysis. Indeed, the PCR has been successfully used to amplify target DNAs for mutation analysis, genetic mapping and sequencing. However, before subsequent analysis of the amplicon can occur, one must purify the PCR product to remove unwanted salts, primers and enzymes. Numerous strategies have been used to perform purification of PCR-generated DNA. These include gel filtration [1], gel electrophoresis [2], ultrafiltration [3] and ion exchange [4,5]. However, these processes require large amounts of solvents, can be time-consuming, plagued by low sample recoveries and often cannot be easily automated. In addition, the gel-based methods require the amplicon to be excised from the gel matrix following separation.

An attractive alternative to the aforementioned purification techniques for PCR products is a reversed-phase separation, which can readily purify PCR products with high recoveries, typically exceeding 97% [6–9]. In addition, the use of reversed-phase chromatography is conducive to automation, making it amenable to implementation in high-throughput

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DNA sequencing applications. For most reversed-phase separations of DNA amplified via PCR, an ion-pairing agent is used in conjunction with a packed-column containing a C_{18} stationary phase. The common ion-pairing agents that have been used are tertiary or quaternary ammonium salts, such as triethylammonium acetate or tetrabutylammonium phosphate.

The use of conductivity detection has been explored in numerous applications, for example, the detection of metals and other ionic materials separated by liquid chromatography and/or capillary electrophoresis (CE) [10–17]. The pioneering work of Mikkers et al. [18] demonstrated the utility of conductivity to detect carboxylic acids using a ''potential gradient'' detector that essentially measured changes in conductance. Since that time, several different conductivity detector designs have been reported for a variety of separation platforms [13,14,19]. However, to-date, no reports have appeared describing the use of conductivity detection in reversed-phase liquid chromatography (RPLC) for the separation and analysis of oligonucleotides, such as PCR products.

Conductivity can potentially offer several advantages compared to other common detection schemes used for DNA detection in conjugation with the micro-column separation methods, such as UV absorbance or fluorescence. UV absorbance suffers from poor detection limits, which have been reported to be on the order of 10^{-4}–10^{-5} *M* range for DNAs [20] when the separation is performed using micro-columns because of the reduced pathlength associated with the column. The advantage of conductivity detection is that it is amenable to small column detection. For conductivity detection, the analytical response (*G*, conductivity) is related to the concentration of the species being interrogated through the relationship:

$$G = \frac{(\lambda_+ + \lambda_-)C}{1000K}$$

where λ_+ and λ_- are the limiting ionic conductance's of cations and anions in solution, respectively (S cm^2 equiv.$^{-1}$, S = siemens), *C* is the concentration (*M*) and *K* is the cell constant. The cell constant is calculated from *L*/*A*, where *L* is the distance between the electrode pair and *A* is the electrode area. As can be seen from this relation, a small electrode spacing will increase the sensitivity of the measurement making this detection strategy particularly attractive for miniaturized separation platforms. While fluorescence methods can demonstrate low limits of detection (LODs), the difficulty with the strategy is that staining or labeling of the oligonucleotide is performed prior to detection [21]. As a result, pre-sample processing is required and if the purification product is used for further analysis, destaining of the target is necessary as well. Since conductivity measures the bulk conductance of the solution, as long as the analyte has a conductance different from that of the carrier mobile phase solution, the DNA can be analyzed in its native state. In addition, the equipment required for implementation of conductivity can be much simpler and inexpensive compared to laser-induced fluorescence.

The work described in this manuscript outlines the development and operational characteristics of a novel, miniaturized PCR separation/purification system, which utilizes ion-pairing RPLC for the separation with bipolar-pulsed conductivity detection. The separation was performed using a micro-column packed with a C_{18} stationary phase and a mobile phase comprised of water–CH_3CN (50:50) with triethylammonium acetate as the ion-pairing agent.

2. Experimental

2.1. Construction of the conductivity cell

A schematic of the conductivity cell is shown in Fig. 1. The conductivity cell was constructed from Pt wires (Scientific Instrument Services, Ringoes, NJ, USA), where each wire was 360 μm in diameter. Two pieces of Pt wire were cut to 2.5 cm in length and the end of the wire polished with fine grit sandpaper. The ends of the wires were inspected under a 20× stereomicroscope to insure proper polishing. After polishing, the wires were cleaned with isopropyl alcohol then inserted into opposite ends of a glass tee (Innovaquartz, Phoenix, AZ, USA), which had an inside diameter of 365 μm. The ends of two pieces of 75 μm I.D. fused-silica capillary were then polished and a piece of 60 μm I.D. tungsten wire inserted into the capillaries con-

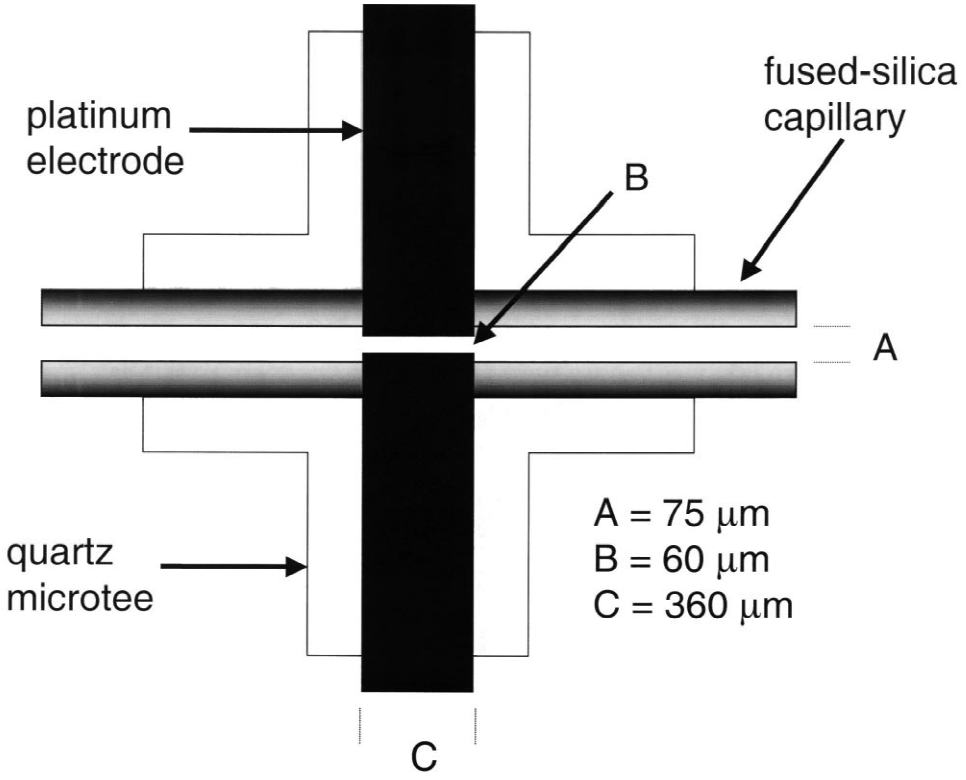

Fig. 1. Schematic of the conductivity cell.

necting them. The capillaries were then inserted into the tee, where the tungsten wire served as an alignment tool for the Pt wires. The Pt wires were pushed against the tungsten wire and glued into place with Bond It adhesive (Scientific Instrument Services). Then, the fused-silica capillaries were pushed against the Pt wire and tacked into place. The tungsten wire was then simply pulled from the capillaries leaving a 60 μm spacing between the Pt wires.

The bipolar-pulsed technique for conductance measurements was used for obtaining the solution conductivity [13,22,23]. The electronics were controlled by a personal computer operating at 5 MHz. The bipolar pulse waveform was controlled by a 12 bit digital-to-analog (D/A) board (National Instruments, Austin, TX, USA). A brief pulse (pulse width = 100 μs) of positive polarity was followed by a negative pulse of the same time duration and amplitude. The use of the bipolar pulse technique allows the ability to discriminate against the cell double layer charging current from the current flowing between the electrode pair when a fixed voltage is applied, which can be related to the solution conductivity through Ohm's law. The bipolar pulse height was controlled by the D/A output and was limited by the resolution of the D/A conversion. The frequency of the pulses (typically 5000 Hz) was controlled by a timer on the D/A board. The timer output was connected to a standard digital input port. Each time the computer detected a rising edge from this input port it generated the bipolar pulse. The potential of one cell electrode was maintained at virtual ground while the potential of the other electrode was controlled by the generated waveform. The current, which was sampled at the end of the negative pulse, flowed through a feedback resistor and was processed by an amplifier and subsequently reported at an analog output pin. Software was written in LabView (National Instruments).

2.2. Flow injection analysis (FIA)

A flow injection system was built in the laboratory to characterize the conductivity detector. It consisted of a HPLC pump (Rainin Instruments, Woburn, MA, USA) connected to a standard 75 μm I.D. fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) which was connected to the inlet of the glass tee that housed the conductivity detector. All flow injection studies were run at 5 μl/min flow-rates. KCl standard solutions and Herring Sperm DNA (Gibco-BRL, Gaithersburg, MD, USA) standard solutions were prepared in 18 MΩ doubly-distilled water (ddH_2O) daily for characterization studies.

2.3. μ-RP-HPLC system

A Rainin Rabbit HP pump (Rainin Instruments) was used to pump the mobile phase through the system. One end of a 15 cm×300 μm I.D. reversed-phase Hypersil (C_{18}) column (ThermoQuest, Hollis-ton, MA, USA) was connected to a low-volume injector (Valco Instruments, Houston, TX, USA) which was connected to the high-pressure mobile phase pump. The low-volume injector allowed re-producible injection of 100 nl volumes of sample into the μ-RP-HPLC system.

The chromatography of the PCR product was carried out using ion-pairing RPLC with the mobile phase consisting of acetonitrile–water (50:50) (pH 7.0) and 50 m*M* triethylammonium acetate (TEAA). The volumetric flow used was 7.5 μl/min and the conductivity cell was operated at a frequency of 5000 Hz with a 0.5 V bias voltage applied between the electrode pair. The PCR mix was manually loaded into the low-volume injector to inject sample into the flow stream of the μ-RP-HPLC system. Also, the PCR product was compared to a DNA low mass sizing ladder (Gibco-BRL) that contained five fragments of 200, 400, 800, 1000 and 1200 base pairs (bp). The sizing ladder was separated using the separation conditions described above. All resolution (R_s) values were calculated based on the following formula:

$$R_s = \frac{t_{r_2} - t_{r_1}}{w_{1/2_2} + w_{1/2_1}}$$

where t_r is the retention time of the components (in units of time) and $w_{1/2}$ is the width of the corresponding peaks at half height.

2.4. PCR amplification of λ-bacteriophage DNA

A 500-bp fragment of λ DNA (5.0 ng/μl) (Gibco-BRL) was amplified by PCR in a Perkin-Elmer 2400 series thermocycler (Foster City, CA, USA). The PCR mixture contained 1 μl of λ DNA, 10 μl 1× PCR buffer (20 m*M* Tris–HCl, pH 8.4, 50 m*M* $MgCl_2$), 2 μl deoxynucleotides (dNTPs), 85 μl ddH_2O, 1 μl PCR01 forward primer (1 μ*M*, 5′-GATGAGTTCGTGTCCGTACAACTGG-3′) and 1 μl of PCR02 reverse primer (1 μ*M*, 5′-GGTTAT-CGAAATCAGCCACAGCGCC-3′). PCR was performed under ''hot start'' conditions, which entailed addition of 1 μl of the *Thermus aquaticus* (Taq) DNA polymerase (Gibco-BRL) after the reaction temperature reached 80°C, which ensured high fidelity during DNA amplification. Twenty-five and 30 PCR cycles were performed using the following program: (1) denature double-stranded (ds) DNA at 94°C for 45 s; (2) anneal primer at 55°C for 30 s and (3) extend primer at 72°C for 90 s.

2.5. PCR amplicon sizing

Following amplification, sizing of the 500-bp PCR amplicons was accomplished by slab-gel electrophoresis using an 0.8% agarose gel. Fig. 2 shows the gel electropherogram of the PCR product along with the sizing ladder. The amplicons were run in parallel with a DNA low mass sizing ladder (Gibco-BRL) to assure the integrity of the PCR reaction.

3. Results and discussion

3.1. Characterization of the conductivity detector using FIA

In order to determine the analytical figures of merit of conductivity detection for DNAs (linearity of response and concentration/mass limit of detection), experiments were performed using FIA. Various concentrations of KCl and DNA were injected into the system and the conductivity of the

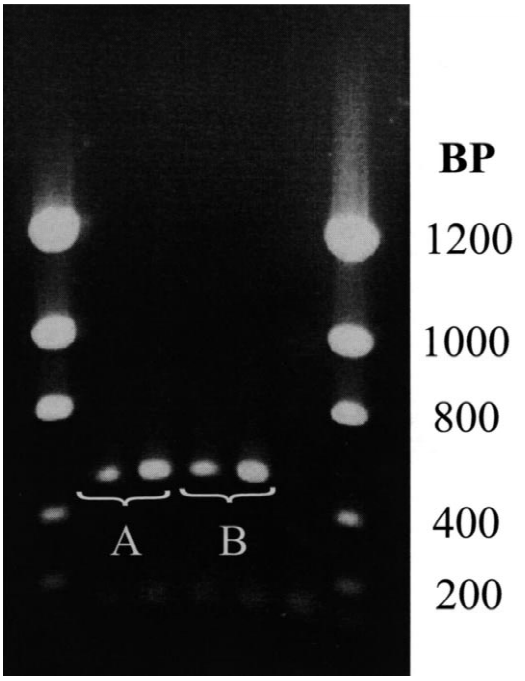

Fig. 2. Slab gel electropherogram of (A) 25 cycle and (B) 30 cycle PCR mixes run in parallel with a low DNA mass sizing ladder. The number of bases for each fragment in the sizing ladder is labeled on the side of each band. The sieving matrix (gel) consisted of 0.8% (w/v) agarose with ethidium bromide used as the staining dye. The running buffer consisted of 100 m*M* Tris–borate–EDTA (TBE), pH 8.3.

sample was measured against ddH_2O and 50 m*M* TEAA carrier solution. The choice of carrier solution was based upon the fact that it would serve as the ion-pairing mobile phase in the μ-RP-HPLC separation. It was important to measure the observed response against the mobile phase used for μ-RP-HPLC in these FIA studies, since the conductivity response results from a differential measurement between the carrier solution and that of the sample. As such, the background conductivity will have a profound influence on the analytical signal with a higher background conductivity associated with the mobile phase degrading the signal-to-noise ratio (*S*/*N*) in the measurement. However, this must be balanced with the chromatographic needs, since the ion-pairing agent concentration will affect the resolution in the chromatography of the PCR product. Calibration plots (see Fig. 3) were constructed from the data for both KCl and DNA by integrating the area under each FIA peak and averaging over five runs. The concentration range for the DNA sample was from 0.50 to 2.50 mg/ml, while for KCl it was from 1.00 to 100 μg/ml. Each plot was fit to a straight line and the linear correlation coefficients were found to be 0.9971 for the DNA sample and 0.9991 for KCl. By extrapolating the plots to 0 concentration using a $S/N=3$ criteria, the detection limits were calculated for DNA and KCl. A LOD of 110 mg/l (330 n*M*) was calculated for the DNA sample, which is approximately two-orders of magnitude lower than LODs reported for UV absorbance detection using a 75 μm I.D. capillary column [20,24]. The LOD for KCl was found to be 34.6 μg/l. Taking into account the injection volume (100 nl), the mass detection limits were 11.0 ng and 3.46 pg for the DNA and KCl samples, respectively. The improved mass LOD for KCl results from the fact that its conductivity is significantly different from that of the carrier solution. For comparison to fluorescence, the mass detection limit of dsDNAs stained with mono-intercalating dyes and separated by CE has been reported to be 20 fg [21]. While the mass detection limit obtained for the conductivity detector is not as good as fluorescence, conductivity is much easier to implement due to the fact that staining is not required and also, the instrumentation is significantly less complex. It should be noted that improvements in the mass LOD could also be realized by lowering the conductivity of the carrier solution. Therefore, if the counter ion concentration, TEAA in this case, could be reduced, improvements in the LOD could be achieved because the mode of detection is an indirect mode. For indirect detection it has been shown that the concentration limit of detection is directly proportional to the counter-ion concentration [25]; thus by reducing the counter ion concentration the concentration LOD is improved.

3.2. Separation of PCR fragment

After amplification, conductivity detection of a 500-bp segment of λ DNA was tested using the conductivity cell. The amplification reaction was cycled 25 or 30 times and analyzed using ion-pair on a reversed-phase (C_{18}) micro-column. The results of the PCR analyses are shown in the chromatograms of

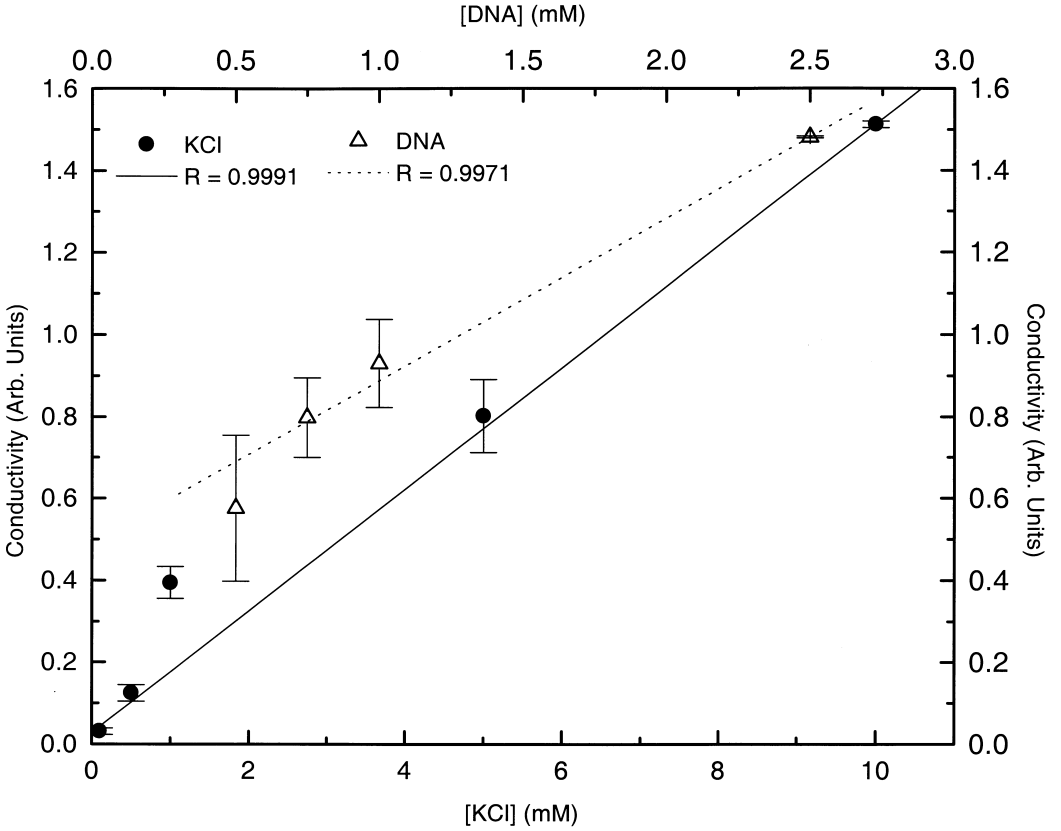

Fig. 3. Calibration plots for KCl (●) and DNA (△). The plots were constructed by integrating the area under each FIA peak and averaging over five runs. The KCl was run using ddH_2O as the background carrier and the DNA was run using 50 m*M* TEAA as the background carrier.

Fig. 4. The primer set forms a primer–dimer product during amplification, which co-elutes in the unretained peak in this separation and is also comprised of the buffer and metal ions used in the PCR cocktail. This was determined by performing a null PCR with all PCR ingredients thermal cycled except the target DNA and then, simply performing a separation on the μ-RP-HPLC system (data not shown). Indirect detection of the PCR amplicon was observed, which caused a negative peak to occur when the amplicon passed the conductivity cell because the DNA had a lower conductivity than the background carrier (see Fig. 4B and C). Also, the PCR results were compared to a standard DNA ladder (Fig. 4A) for sizing purposes. The separation of the standard ladder was performed under the same conditions as that for the PCR amplicon. As can be seen in Fig. 4A, the 200/400 and 400/800 bp fragments were baseline resolved ($R_s \geq 1.5$) with resolution values being 1.69 and 1.98, respectively. The 800/1000 and 1000/1200 bp fragments were only partially resolved with the resolution factors determined to be 1.13 and 1.01, respectively. It should also be noted that the elution time of the PCR product matched the appropriate fragment on the sizing ladder indicating the correct identity of this product (500 bp). Although the components of the ladder were not completely resolved, baseline resolution of the ladder could be accomplished by simply adjusting the running buffer composition as shown by previous work using ion-pairing chromatography [26]. However, changes in the ion-pairing concentration will affect the analysis (plate numbers, elution time, etc.) for the PCR amplicon as well.

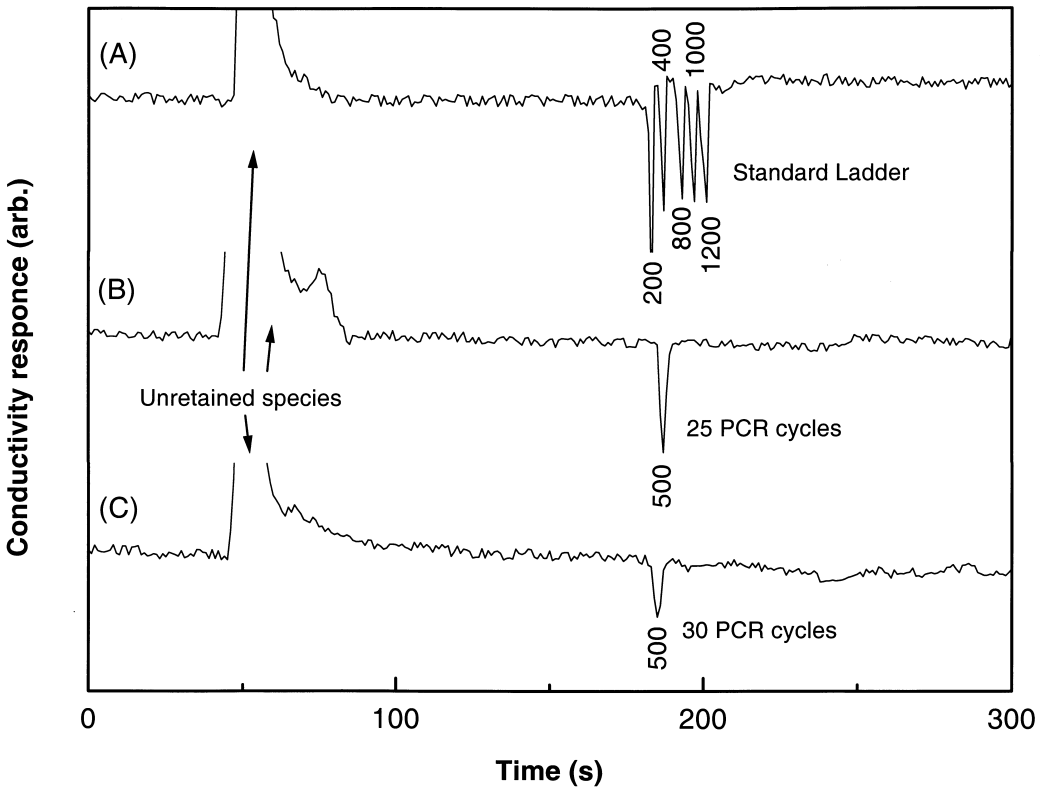

Fig. 4. Chromatograms of (A) a standard low mass DNA ladder, (B) 25 cycle and (C) 30 cycle 500 bp PCR product separated by μ-RP-HPLC. The mobile phase composition was water–acetonitrile (50:50) with 50 m*M* TEAA (pH 7.0). The injection volume was 100 nl and the flow-rate was 7.5 μl/min. The bias voltage applied to the conductivity cell was 0.5 V.

Table 1 shows a list of chromatographic and detection efficiencies. The concentration of the 500-bp λ DNA in each series of PCRs was estimated by interpolation of the calibration plot of concentration vs. peak area for herring sperm DNA. The estimated values of 1.8 mg/ml (25 cycles) and 2.1 mg/ml (30 cycles) corresponds to an increase of $3.6 \cdot 10^6$ and $4.2 \cdot 10^6$, respectively, over the initial concentration of 0.5 ng/ml for the target. After accounting for the injection volume (100 nl), each injected sample contained $3.3 \cdot 10^{11}$ and $3.8 \cdot 10^{11}$ copies of the 500-bp PCR amplicon for the 25 and 30 PCR cycles, respectively. The theoretical copy number (N) for 25 PCR cycles can be calculated from $N = N_0 2^n$, where N_0 is the starting copy number and n is the number of PCR thermal cycles. For the conditions used for our PCRs, we would expect to see $1.53 \cdot 10^{11}$ copies in a 100 nl volume after 25 PCR cycles, in close agreement to the number obtained from the calibration plot. The reason that the copy number is not increased by 32-fold (2^5, where 5 is the difference in PCR cycle number between the two samples) for the 30 cycle PCR mix is that we are most likely operating on the plateau region of the amplification versus cycle number, where the copy number does not scale with the cycle number [27] due to deactivation of the polymerase enzyme either through heat deactivation or production of large amounts of DNA.

Table 1
Chromatographic efficiencies, LODs and amplification numbers for PCR amplified λ DNA

	25 PCR cycles	30 PCR cycles
k'	2.48	2.51
Plate numbers (N)	5888	9098
Mass of DNA detected (ng)	180	210
No. of DNA copies ($\cdot 10^{11}$)	3.3	3.8

As can be seen from Table 1, the plate numbers

were found to be 5888 (25 thermal cycles) and 9098 (30 thermal cycles), which were calculated assuming the peaks were Gaussian-shaped (i.e., no asymmetry). For the 30-thermal cycle case, the height equivalent to a theoretical plate (HETP) value was determined to be $1.65 \cdot 10^{-3}$ cm, yielding a total variance (σ_t^2) of $2.48 \cdot 10^{-2}$ cm^2. Calculation of the variance arising from the conductivity detector alone produced a value of $1.08 \cdot 10^{-4}$ cm^2, only a 0.44% contribution to σ_t^2 indicating that the major contribution to the band broadening is kinetic effects associated with the column. The additional extra-column variances, for example the variance arising from the injection volume or connecting tubing, were calculated to be $<0.1\%$ of the total variance. In addition, the resolution value between the amplicon peak and void volume, which consisted of the PCR cocktail reagents such as salts, dNTPs, primers and enzyme, was found to be 11.33, with a capacity factor of 2.5 for the PCR amplicon. The large resolution factor observed for this separation will allow for the efficient isolation of the amplicon for potential down-stream processing applications, such as sequencing. Removal of excess PCR reagents is necessary to obtain high-quality sequencing reads for both slab and capillary gel separations [28,29].

4. Conclusions

The feasibility to perform separation and/or purification of PCR products in a μ-RP-HPLC format using ion-pairing agents with conductivity detection has been demonstrated. One of the advantages of μ-RP-HPLC is that very low sample volumes and mobile phase volumes compared to conventional PCR separation/purification methods are required. For example, during the ~4 min run used to isolate the PCR amplicon in this work, only 30.00 μl of mobile phase was consumed (flow-rate = 7.5 μl/min). In addition, we have demonstrated the utility of conductivity detection for the monitoring of oligonucleotides in chromatographic applications. Since the analytical response is produced from a difference in conductivity between the carrier solution and the sample, no staining of the sample was required. Therefore, the detection process is non-destructive and allows the target to be readily accessible for further analysis without significant post-run processing. Furthermore, this detection scheme is very amenable to miniaturization since the response sensitivity is improved when the electrode spacing is reduced compared to UV absorbance, which shows poor S/N values in micro-columns due to the pathlength dependent signal response. This will make conductivity particularly appealing for micro-column separation platforms, such as CE and microchip electrophoresis.

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References

[1] M. Kreoitman, L.F. Landweber, Genet. Anal. Tech. 6 (1989) 84.
[2] G. McMahon, E. Davis, G.N. Wogan, Proc. Natl. Acad. Sci. USA 84 (1987) 4974.
[3] M. Mihovilovic, J.E. Lee, BioTechniques 7 (1989) 14.
[4] E.D. Katz, L.A. Haff, R. Eksteen, J. Chromatogr. 512 (1990) 433.
[5] W. Warren, J. Doniger, BioTechniques 10 (1991) 216.
[6] P. Gold, BioTechniques 13 (1992) 132.
[7] A.V. Vordam, J. Kerschner, Anal. Biochem. 152 (1986) 221.
[8] C.A. Robert, J.L. Yang, R. Wu, Methods Enzymol. 68 (1979) 176.
[9] Y. Kato, M. Sasaki, T. Hashimoto, T. Murotsu, S. Fukushige, K. Matsubara, J. Biochem. 95 (1984) 83.
[10] T.N.G. Tsuda, M. Sato, K.J. Yagi, J. Appl. Biochem. 5 (1983) 330.
[11] P. Gebauer, M. Deml, P. Bocek, J. Janak, J. Chromatogr. 267 (1983) 455.
[12] F. Foret, M. Deml, V. Kahle, P. Bocek, Electrophoresis 7 (1986) 430.
[13] S. Kar, K.D. Purnendu, H. Liu, H. Hwang, Anal. Chem. 66 (1994) 2537.
[14] X. Huang, M.J. Gordon, R.N. Zare, J. Chromatogr. 425 (1988) 385.
[15] X. Huang, T.J. Pang, M.J. Gordon, R.N. Zare, Anal. Chem. 59 (1987) 2747.
[16] M. Deml, F. Foret, P. Bocek, J. Chromatogr. 320 (1988) 159.
[17] J. Beckers, T. Verheggen, F. Everaerts, J. Chromatogr. 452 (1988) 591.

[18] F. Mikkers, F. Everaerts, T.J. Verheggen, J. Chromatogr. 169 (1979) 1.
[19] P.K. Dasgupta, L. Boa, Anal. Chem. 65 (1993) 1003.
[20] P. Singhal, W.G. Kuhr, Anal. Chem. 69 (1997) 3552.
[21] C.V. Owens, Y.Y. Davidson, S. Kar, S.A. Soper, Anal. Chem. 69 (1997) 1256.
[22] D.E. Johnson, C.G. Enke, Anal. Chem. 42 (1970) 329.
[23] K.J. Caserta, F.J. Holler, S.R. Crouch, C.G. Enke, Anal. Chem. 50 (1978) 1534.
[24] H. Arakawa, S. Nakashiro, A. Tsuji, M. Maeda, J. Chromatogr. B 716 (1998) 119.
[25] E.S. Yeung, W.G. Kuhr, Anal. Chem. 63 (1991) 275A.
[26] S. Eriksson, G. Glad, P. Pernemalm, E. Westman, J. Chromatogr. 359 (1986) 265.
[27] C.T. Wittwer, M.G. Merrman, A.A. Moss, R.P. Rausmussen, BioTechniques 22 (1997) 130.
[28] J. Zimmerman, H. Voss, S. Wiemann, T. Rupp, N.A. Hewitt, C. Schwager, J. Stegmann, W. Ansorge, Methods Mol. Cell. Biol. 4 (1993) 29.
[29] A. Rosenthal, O. Coutelle, M. Craxton, Nucleic Acids Res. 21 (1993) 173.