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Quantitation of polymerase chain reaction products by capillary electrophoresis using laser fluorescence☆

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Abstract

In samples where the amount of DNA is limited, the polymerase chain reaction (PCR) can amplify specific regions of the DNA. A quantitative analysis of the PCR product would be desirable to ensure sufficient DNA is available for analysis. In this study, we examine the use of capillary electrophoresis (CE) with laser fluorescence detection for quantitation of PCR products. A coated open tubular capillary was used with a non-gel sieving buffer and a fluorescent intercalating dye to obtain results within 20 minutes. Using an internal standard, peak migration time was below 0.1% relative standard deviation (R.S.D.) with a peak area precision of 3% R.S.D. In comparison to quantitation by hybridization, (i.e., slot blot) and spectrophotometric analysis, capillary electrophoresis shows distinct advantages due to its ability to separate unincorporated primers and PCR byproducts from the targeted PCR product. The results demonstrate that CE can be used to monitor the quality and quantity of the PCR product.

1. Introduction

With the advent of the polymerase chain reaction (PCR) almost a decade ago, specific regions of low-copy DNA can be amplified to a level where they can be detected more readily [1,2]. Since the efficiency of PCR may vary from reaction to reaction, in some cases such as sequencing, it may be necessary to monitor the quality and quantity of the amplification [3,4].

The most common method of PCR product analysis involves gel electrophoresis and subsequent staining for detection purposes [3–6]. Quantitation by immobilization of the DNA to a support membrane followed by hybridization with chemiluminescent or radioactively tagged oligonucleotide probes (i.e. slot blot) [7] or fluorescence spectrophotometry (i.e. fluorometry) [8,9] are often used to determine how much amplified DNA is in a sample. Although the hybridization technique provides some qualitative information as opposed to fluorometry, it remains standard practice to use these methods in conjunction with conventional gel electrophoresis to distinguish targeted PCR products from PCR primers and contaminants. While DNA detection and quantitation using the hybridiza-

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tion method may be automated, it is not routine practice in many laboratories. To date, automation of fluorometric assays is not routinely done.

Capillary electrophoresis (CE) has become a useful analytical technique in the past few years and has the potential for quantitation of DNA [10–13]. CE can provide fast, efficient separations of small ions and biologically active molecules such as proteins and DNA. Moreover, sample injection can be automated. Computer storage of electropherograms facilitates easy database formation and retrieval.

Unfortunately, injection variation and inadequate standards have kept quantitation of biological samples by CE from being applied routinely [14]. However, quantitation of nucleic acids by capillary electrophoresis has been described recently by several research groups [15–17]. Using ϕX-174 DNA-*Hinc*II digest as an internal standard, Nathakarnkitkool et al. [15] analyzed a 361-bp segment of an androgen receptor mRNA transcript in less than 20 min with a peak area relative standard deviation (R.S.D.) of 3.06% ($n = 6$). This work has been accomplished by using restriction digests as internal standards. Schwartz and coworkers [16] obtained migration time precision of less than 0.2% R.S.D. with DNA restriction fragments; the area precision for seven injections at 10 μg/ml of ϕX 174 *Hae*III digest was 1.91–8.43% R.S.D. The problem with the above procedures is that the ideal standard for DNA analysis should contain a single peak.

In the current paper, we demonstrate the feasibility of capillary electrophoresis for PCR product quantitation. Using internal standards of known size and concentration, the issues of detection limits, precision, and linear range are examined, and direct comparisons of results obtained from other quantitation methods are presented.

2. Experimental

2.1. DNA samples used

*Hae*III digested ϕX 174 DNA (Sigma, St. Louis, MO, USA) was diluted to the required concentration using deionized water. K562 cell line DNA was obtained from Life Technologies, (Gaithersburg, MD, USA) and diluted in 10 m*M* Tris-HCl, 1 m*M* EDTA (TE) buffer. Mitochondrial DNA (mtDNA) samples were extracted from human hair and blood samples [18].

2.2. Internal standards

Internal standards were PCR products generated by BioVentures (Murfreesboro, TN, USA). The M100, M200, and M400 (100 bp, 200 bp, and 400 bp size fragments, respectively) were used. The standards were quantitated by fluorescent spectrophotometry and diluted to 0.576 ng/μl (100 bp) and 0.896 ng/μl (200 bp) with deionized water. From these stock solutions, 34 μl were placed into the CE sample vial after which 1 μl of PCR product was added and mixed with a pipet.

2.3. PCR amplification

Three different amplifications were performed on two hypervariable areas of the control region of human mtDNA [19]: HV1A (280 bp in length), HV1B (276 bp in length), and HV2 (416 bp in length). Primers used included: HV1A = L16016 (5′-CAC CAT TAG CAC CCA AAG CT-3′) and H16255 (5′-CTT TGG AGT TGC AGT TGA TGT-3′); HV1B = L16159 (5′-TAC TTG ACC ACC TGT AGT AC-3′) and H16395 (5′-CAC GGA GGA TGG TGG TCA AG-3′); and HV2 = LO34 (5′-CAC CCT ATT AAC CAC TCA CG-3′) and H408 (5′-CTG TTA AAA GTG CAT ACC GCC A-3′).

PCR was performed in 0.2-ml MicroAmp reaction tubes (Perkin Elmer, Norwalk, CT, USA) with 200 μM each dATP, dCTP, dGTP, and dTTP; 1 μM of each primer (two per reaction; see above); 50 m*M* KCl, 10 m*M* Tris-HCl pH 8.3, 1.5 m*M* $MgCl_2$, 0.001% gelatin, and 4 μg BSA (Sigma); and 5.0 units/reaction of AmpliTaq DNA Polymerase (Perkin Elmer). Amplifications were performed in 25-μl volumes using the Perkin Elmer GeneAmp PCR System 9600 thermal cycler and the following parameters: 1 min 95°C initial denaturation followed by

36 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s.

2.4. Post-PCR sample preparation and cleanup

The primers were removed from the amplified DNA by centrifuging the sample in Microcon-100 tubes (Amicon, Beverly, MA, USA). Three 300-μl washes with deionized water were performed using centrifugation at $1000 \times g$ for 5 min. After adding 15 μl of deionized water to the filter, the tube was inverted and centrifuged at 1000 g for 5 min. A retentate of approximately 20 μl was collected. A 1-μl volume of this retentate, which contained the recovered PCR product, was added to 34 μl of deionized water containing an internal standard.

Further dialysis to remove salts was carried out by pipetting the retentate onto a 0.025-μm filter (Millipore Type VS) which was floating in a Petri dish filled with deionized water. This float dialysis was performed for 30 min [20]. The removal of interfering salts and primers through the Microcon and Millipore dialysis facilitates sample introduction onto the column when applying electrokinetic injection [16]. This additional dialysis step was not required when hydrodynamic injection was utilized.

2.5. CE system

The capillary electrophoresis instrument used in this study was the Beckman P/ACE System 2050 (Beckman Instruments, Palo Alto, CA, USA) with a Laser Module 488 argon-ion laser. DB-17 coated capillaries (J&W Scientific, Folsom, CA, USA) with 50 or 100 μm I.D. and 57 cm lengths (50 cm effective length) were employed. The coating thickness was 0.1 μm. An optical window was produced by etching a short section of the polyimide coating with hot fuming sulfuric acid (Aldrich). The electrophoretic buffer consisted of 1.0% (w/v) hydroxyethyl cellulose (Aldrich Cat. No. 30, 863-3), 100 mM Tris-borate, 1 mM EDTA, 1.27 μM ethidium bromide (Sigma), and 50 ng/ml YO-PRO-1 dye (Molecular Probes, Eugene, OR, USA) [21]. The pH was adjusted to 8.7 or 8.9 with CsOH. Separation took place at 20°C or 25°C with constant voltage separation at −15 kV.

Two different injection schemes were used, hydrodynamic and electrokinetic. The hydrodynamic injections consisted of a 10-s injection of water followed by a 45-s injection of sample. All pressure injections were performed at 3.44 MPa (0.5 psi). For electrokinetic injection, an electric potential of 5 kV was applied to the sample for durations ranging from 1–30 s depending on the sample concentration [22].

Laser-induced fluorescence (LIF) was achieved by the excitation of the intercalated YO-PRO-1 dye–DNA complex with the argon-ion laser 488 nm line and detection of emitted light at 520 nm. *Hae*III digested ϕX 174 DNA was used to test column resolution. If the 271 bp and 281 bp peaks of the digest were resolved, then analysis proceeded.

The column was stored overnight in water and rinsed each morning for 20 min with HPLC-grade methanol and 20 min with run buffer. Between each run a 4-min MeOH and 6-min run buffer wash were performed. With proper rinsing, columns generally last over 100 runs without a significant loss of resolution.

Data were collected and the peaks integrated on Millennium 2010 (Waters Chromatography, Milford, MA, USA). The migration time, migration time ratio (of the sample to the internal standard), area, height, and adjusted area (area divided by migration time) were recorded for each sample component.

2.6. Fluorescent spectrophotometric analysis

A Perkin Elmer 650-40 fluorescent spectrophotometer was used following the procedure described by Rye et al. [8]. YOYO (the homodimer of YO-PRO, Molecular Probes) was the fluorescent dye used for quantitation. YOYO dye was diluted 1:5000 in TE buffer. For each sample, 5 μl of the PCR product were added to 2 ml of the YOYO-TE buffer solution. K562 DNA was diluted to 25 ng/μl and used as a reference.

2.7. Quantitation by hybridization

The quantitation by hybridization with chemiluminescent detection (i.e. slot blot) procedure as described by Walsh et al. [7] was used. The mtDNA control region (i.e. D-loop) from K562 DNA was amplified by PCR and purified with a Microcon-100 tube. These PCR products then were quantitated by fluorescent spectrophotometry. From this resulting DNA, standards were prepared in 10 μl of 10 m*M* Tris-HCl, 0.1 m*M* EDTA (TE) buffer by serial dilution to amounts of 40 ng, 20 ng, 10 ng, 5 ng, 2.5 ng, 1.25 ng, 0.625 ng, and 0.312 ng. PCR products were assayed after purification through a Microcon-100 tube. Biotinylated oligonucleotides (HV1B = H16255 = Biotin-5′-CTT TGG AGT TGC AGT TGA TG-3′ and HV2 = L172 = Biotin-5′-ATT ATT TAT CGC ACC TAC GT-3′) at a concentration of 1 pmol/μl were used as the slot-blot probes. After exposure of the membrane to autoradiographic film for 15 min, a visual comparison was made between the sample and the standards.

3. Results and discussion

3.1. Examination of quantitation parameters

Hydrodynamic injection of *Hae*III digested ϕX 174 DNA was performed by using a two-step injection procedure [23]. The injection times were optimized by changing the time of a water injection while holding the sample injection time constant. A short injection of water prior to the injection of the sample provides a low-conductivity zone which aids in sharpening the sample zone [23,24]. This water plug allows more sample to be loaded onto the column without a loss of resolution as the sample components can travel a greater distance before they are focused at the water–buffer interface. When the sample is dissolved in water rather than a run buffer, further zone sharpening can occur [24]. Fig. 1 shows that up to a 10-s water injection prior to the sample can be performed without a loss of resolution. The longer water plug is advantageous as it allows more concentrated samples to be loaded onto the column.

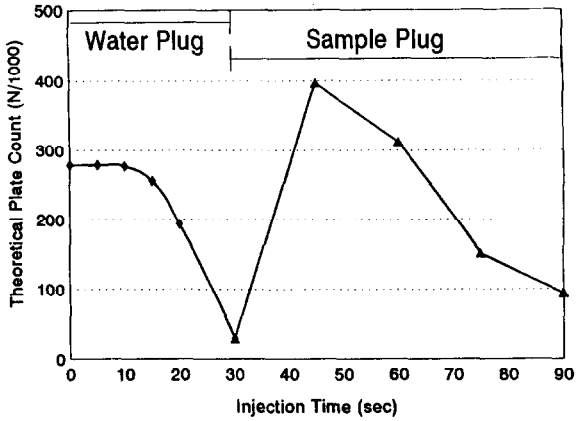

Fig. 1. Plot of number of theoretical plates for 234-bp peak vs. injection time for 2.0 ng/μl of *Hae*III digested ϕX 174 DNA. (◆) water injection variations from 0–30 s; (▲) sample injection variations from 30–90 s. All pressure injections were 0.5 psi (3.44 MPa). Conditions: constant current, 38 μA (−13 kV); temperature, 20°C; LIF detection, 520 nm.

With the water injection time determined, the water injection was then held constant at 10 s while the injection time of the sample was varied between 30 s and 90 s. The highest number of theoretical plates for the 234 bp peak of the ϕX 174 *Hae*III digest sample was obtained with a 10-s water injection followed by a 45-s sample injection (Fig. 1).

With a similar CE system, Ulfelder et al. [23], determined that a pressure injection at 0.5 psi for 11 s produced a plug of water 1 mm long. Using this data we calculate that a 45-s injection introduces approximately 32 nl into the 100-μm I.D. capillary. From these values a minimum detectable concentration of 22 fg/nl (signal-to-noise = 3) can be detected when hydrodynamic injection is used to introduce DNA molecules into the capillary.

Hydrodynamic injection allows direct injection of the sample without prior sample cleanup (i.e. directly from the PCR tube). This can be an advantage when analyzing samples from the PCR which contain added salts and unreacted primers. While electrokinetic injection allows much lower levels of DNA to be determined,

primer removal and dialysis steps are necessary [16]. Excess primers and salt preferentially load onto the column with this injection technique, resulting in poor sensitivity [25].

The migration time precision, peak-area precision, and the linear range were determined using the M100, M200, and M400 standards (BioVentures). Both electrokinetic (EK) and hydrodynamic (HD) injection schemes were followed.

The use of an internal standard with both EK and HD injections in the coated capillaries was found to improve the precision to less than 0.2% R.S.D. Long-term reproducibility was examined with 33 replicate samples of 100 bp, 200 bp, and 400 bp PCR products. The migration time for the 100-bp and 400-bp peaks was measured relative to the 200 bp peak (Table 1). Without the 200-bp peak as an internal standard, the precision dropped from 0.16% to 0.9% for the 100-bp peak and from 0.19% to 0.8% for the 400-bp peak. Short-term reproducibility ($n = 5$) ranged from 0.04% to 0.1% R.S.D.

Because DNA peaks move through the sieving buffer at different rates, each peak needs to be normalized by dividing the area by the migration time. This adjusted area, rather than peak height, is recommended for free zone CE quantitation [14,24,26–28]. Altria [26] showed that decreasing the separation voltage increased the peak area by keeping the peaks in the detection window longer. Table 2 compares the two injection techniques. While electrokinetic injection produces a slightly better peak area and peak-height precision, an internal standard is necessary to adjust for variation in injection conditions [27]. Without an internal standard, hydrodynamic injection is more reproducible.

Peak-area precision was optimized by changing the run buffer vials frequently. The adjusted area for a 100-bp peak ($n = 10$) dropped from 8% R.S.D. to 3% R.S.D. by changing the outlet run buffer vial between every run instead of every five runs (Fig. 2). This result may be due

Table 1
Migration time (MT) precision

	100 bp	400 bp
(A) No internal standard ($n = 33$)		
Average MT (min)	11.7	16.6
R.S.D. (%)	0.9	0.8
(B) Internal standard ($n = 33$)		
MT ratio	0.858	1.223
R.S.D. (%)	0.16	0.19
(C) Internal standard ($n = 5$)		
MT ratio	0.862	1.222
R.S.D. (%)	0.05	0.04

Reproducibility is illustrated with 33 runs containing both HD and EK injections of 100-bp, 200-bp, and 400-bp DNA completed over a two-day period. (A) No internal standard adjustment. (B) Using the 200-bp peak as an internal standard. (C) Short-term reproducibility demonstrated with electrokinetic injection ($n = 5$) and 200-bp internal standard correction. Conditions: 57 cm × 0.1 mm I.D. DB-17 column; 1% HEC, 100 m*M* tris-borate, 1 m*M* EDTA, pH 8.9, 1.27 μM ethidium bromide, 50 ng/ml YO-PRO-1; EK: 3 s, 5 kV; HD: 10 s H_2O, 45 s sample; −15 kV (85 μA); 25°C; LIF detection, 520 nm.

Table 2
Peak area precision

	Hydrodynamic[a]	Electrokinetic[b]
(A) Internal standard		
Adjusted area	8.0%	6.0%
Height	8.1%	3.0%
Migration time	0.1%	0.1%
(B) No internal standard		
Adjusted area	8.4%	28%
Height	8.5%	23%
Migration time	0.2%	0.3%
(C) Internal standard		
Adjusted area	3.0%	
Height	6.7%	
Migration time	0.07%	

(A) 100-bp DNA compared to 200-bp DNA internal standard for 10 runs. Both inlet and outlet run buffer vials were changed after run 5. (B) Without using internal standard. (C) Same as (A) but outlet buffer vial was changed after every run. All results are R.S.D.s.

[a] Hydrodynamic (HD) injection (10 s H_2O, 45 s sample) with other conditions as in Fig. 2. Sample: 80 pg of 100-bp and 200-bp DNA.

[b] Electrokinetic (EK) injection (3 s, 5 kV) with other conditions as in Fig. 2. Sample: 18 pg 100-bp and 29 pg of 200-bp.

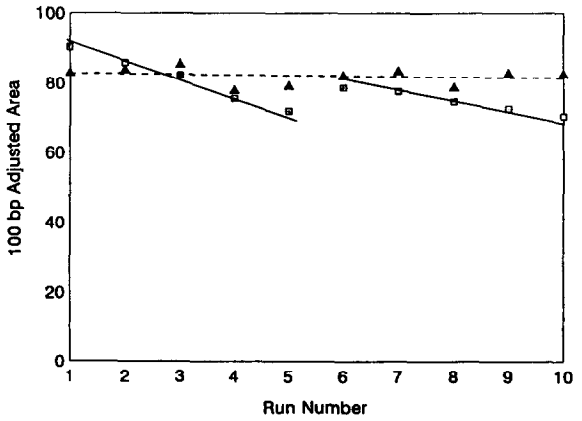

Fig. 2. Adjusted area (peak area divided by migration time) vs. run number for 10 runs. (□) Outlet vial changed after 5 runs. (▲) Outlet vial changed after every run. Sample: 2.5 ng/μl of 100-bp DNA. Conditions: 57 cm × 0.1 mm I.D. DB-17 column; 1% HEC, 100 m*M* trisborate, 1 m*M* EDTA, pH 8.9, 1.27 μ*M* ethidium bromide, 50 ng/ml YO-PRO-1; hydrodynamic injection; −15 kV (85 μA); 25°C; LIF detection, 520 nm.

to the loss of the intercalating dye from the run buffer. This phenomenon suggests that higher concentrations of the intercalating dyes may be required if multiple runs are made without changing the run buffer vials or that run buffer vials should be changed between runs.

A series of 100-bp samples was examined by both HD and EK injection (Fig. 3). As shown in Fig. 3, the HD injection yielded a linear least squares line fit with a correlation coefficient of 0.9991 (A). The linear range with HD injection was from 0.7 pg up to 250 pg. The data illustrate that an internal standard is not necessary when performing an HD injection. An external calibration curve can be run to estimate the concentration of DNA in a sample. The same samples when introduced into the capillary by EK injection displayed quite different behavior as illustrated in Fig. 3B. From 2.5 ng/μl down to 0.156 ng/μl, the samples have nearly the same adjusted area. This indicates that above 0.156 ng/μl the EK injection is independent of concentration. Fig. 3 also illustrates that EK injection has a much lower minimum detectable quantity (MDQ) than HD injection.

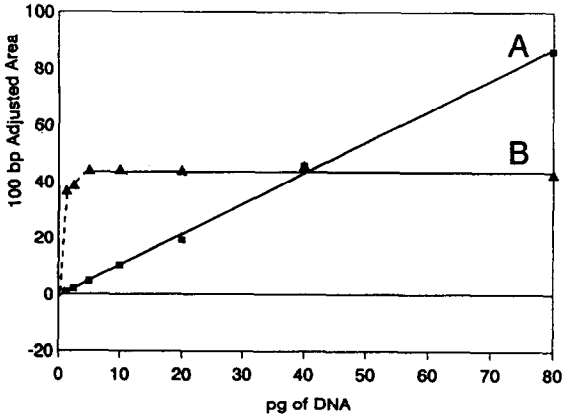

Fig. 3. Adjusted area vs. quantity for 100-bp standard. (A) hydrodynamic injection (■); correlation coefficient (r) = 0.9991. (B) 15 s electrokinetic injection (▲) at 5 kV (26 μA). Conditions as in Fig. 2.

Table 3
Results from DNA quantitation methods

Sample description	CE results[a]	Spec[b]	Hybridization[c]
1. HV1A	30	29.0	ND[d]
2. HV1A	15	17.9	ND
3. HV1A control[e]	below MDQ	2.98	ND
4. HV1A control	below MDQ	5.49	ND
5. HV1B	56	55.1	100
6. HV1B	26	32.5	25
7. HV1B	1.1	5.70	0.6
8. HV1B	2.2	4.25	1.3
9. HV1B control	5.5	4.99	2.5
10. HV1B control	below MDQ	12.6	0.6
11. HV2	3.0	6.97	5
12. HV2	21	23.8	20
13. HV2 control	5.0	18.8	5
14. HV2 control	below MDQ	8.60	0.6

HV1A, HV1B, and HV2 PCR amplified mtDNA samples quantitated by CE–LIF, fluorescent spectrophotometer, and hybridization. MDQ = minimum detectable quantity (~1 ng/μl). All results are listed in ng/μl.

[a] CE conditions as in Fig. 2, except pH 8.7; electrokinetic injection, 5 s at 5 kV (14 μA); −15 kV (48 μA); 20°C.

[b] Fluorescent spectrophotometer conditions as listed in Experimental. Measurements were made in triplicate and averaged.

[c] Hybridization procedure as described in Experimental.

[d] ND = not determined. No slot-blot HV1A probes were available at time of study.

[e] Controls are PCRs run without template DNA.

3.2. CE–LIF results compared to other methods of quantitation

A direct comparison of CE–LIF results with those obtained by other methods demonstrates the quantitative potential of CE (Table 3). In samples 1, 2, 5, 6, 8, 9, and 12, the CE results compare favorably with the fluorescent spectrophotometer. Fluorimetric measurements may be higher than CE results in most of the samples because the primers have not been adequately removed. Since analyses of PCR products based on fluorescence or UV absorption measure total DNA content in the sample, they cannot discriminate between amplified product and excess primers. Consequently, without purification to remove the single stranded DNA primers, accurate conclusions as to the quantity of amplified product may not be made. The results for samples 7, 10, 13, and 14 in Table 3 illustrate this limitation due to primer contamination. At low levels of PCR product, the hybridization and CE results are comparable, while the fluorometric readings are higher. In fact, in all of these samples, the slot blot and CE show similar results. As the hybridization method is specific for the target DNA, a corresponding CE result demonstrates the reliability of the technique.

3.3. Application of CE-LIF quantitation

By adding internal standards to PCR products before and after the filtration cleanup procedure, the loss of DNA during the centrifugation was examined. In one experiment, approximately 45% of the PCR product was lost during the

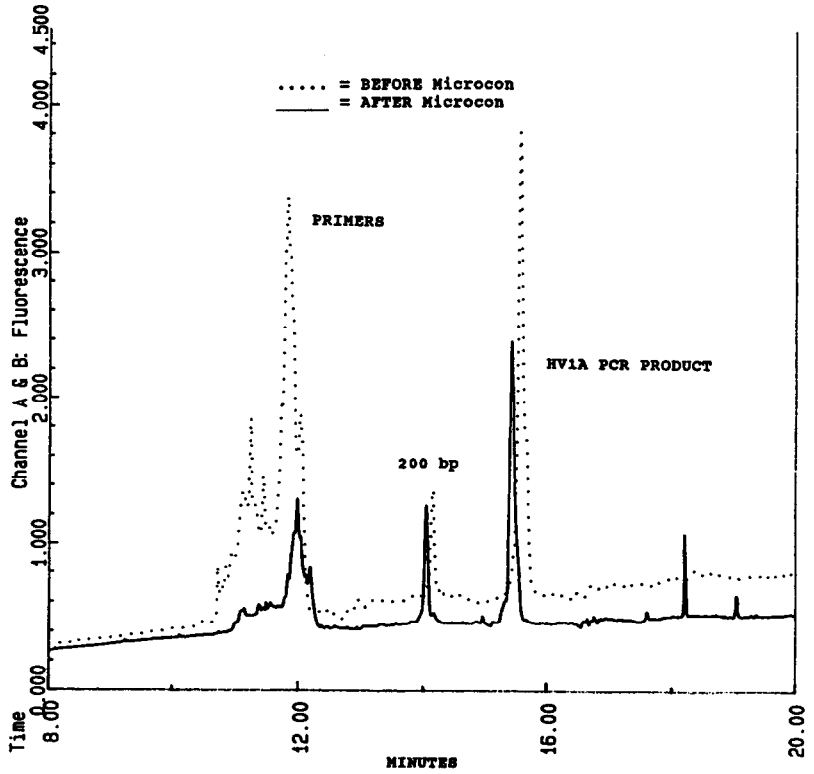

Fig. 4. Electropherogram of 1 μl of HV1A PCR product. The ratio of the product peaks indicates that 45% of the DNA is lost in the cleanup step. BEFORE = 1 μl PCR product prior to filtration added to 34 μl of 200-bp standard. AFTER = 1 μl Microcon 100 retentate added to 34 μl of 200-bp standard. The product peak before cleanup displays that 220 ng/μl of DNA is present. After cleanup, with the same volume of sample, only 120 ng/μl remain. Conditions as in Fig. 2.

filtration process (Fig. 4). The DNA of interest most likely adsorbed to the filter as the concentrated filtrate showed only primer peaks (Fig. 5). Traditional fluorometry and quantitation by hybridization cannot separate and quantitate the individual components of a sample and could not perform this type of analysis. The ability to determine this result highlights one advantage of using CE as a diagnostic tool.

Fig. 6 shows the separation of an HV2 PCR product from excess primers and a nonspecific PCR product. A 200-bp internal standard has been added for quantitation purposes. The ratio of the adjusted product peak area to the adjusted internal standard peak area yields the quantity of the product. The length of the PCR product can be determined by comparing the peak migration time to standards run under similar conditions.

Table 4 compares slab-gel quantitation, fluorescent spectrophotometric analysis, quantitation by hybridization (slot blot), and CE–LIF methods of DNA quantitation. While all of the techniques have advantages and limitations, CE–LIF shows promise because of high sensitivity, good precision, resolution, and current automation capability. Slot blot and fluorometry suffer from the inability to describe the PCR product length or determine PCR byproducts. At present, slab gels and slot blots can run multiple samples simultaneously and thus have a higher throughput. However, further developments in CE such as capillary array electrophoresis will permit higher sample throughput for this technique as well [29].

The sensitivity of laser detection combined with the resolving power of capillary electrophoresis permits minimal sample requirements for PCR product analysis. Thus, more sample is available for sequencing and other post-PCR

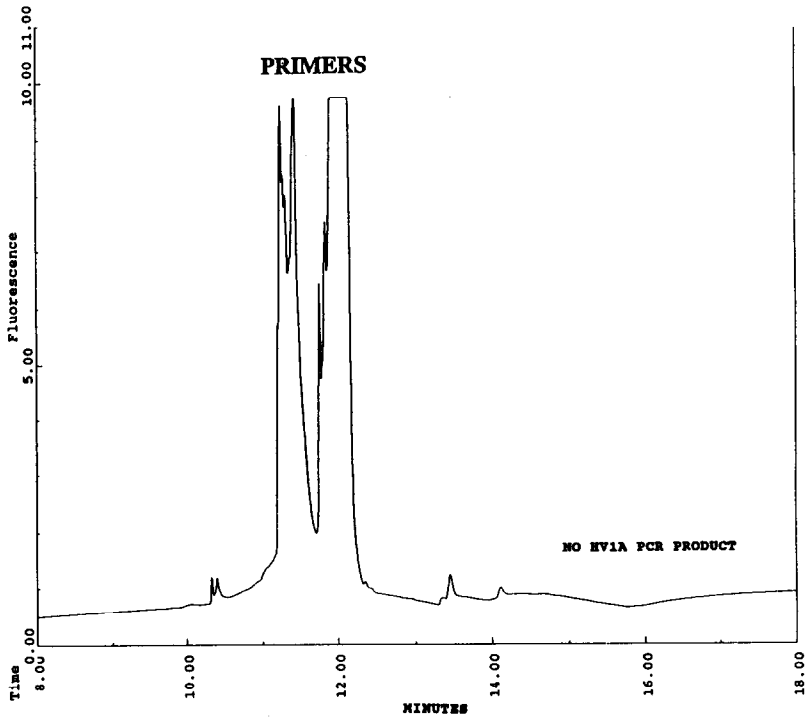

Fig. 5. Electropherogram of concentrated Microcon filtrate. No detectable amount of HV1A PCR product is presented indicating that product may have adsorbed to the filter. Residual primers produce a false signal of 16.0 ng/μl on the fluorescent spectrophotometers. Conditions as in Fig. 2.

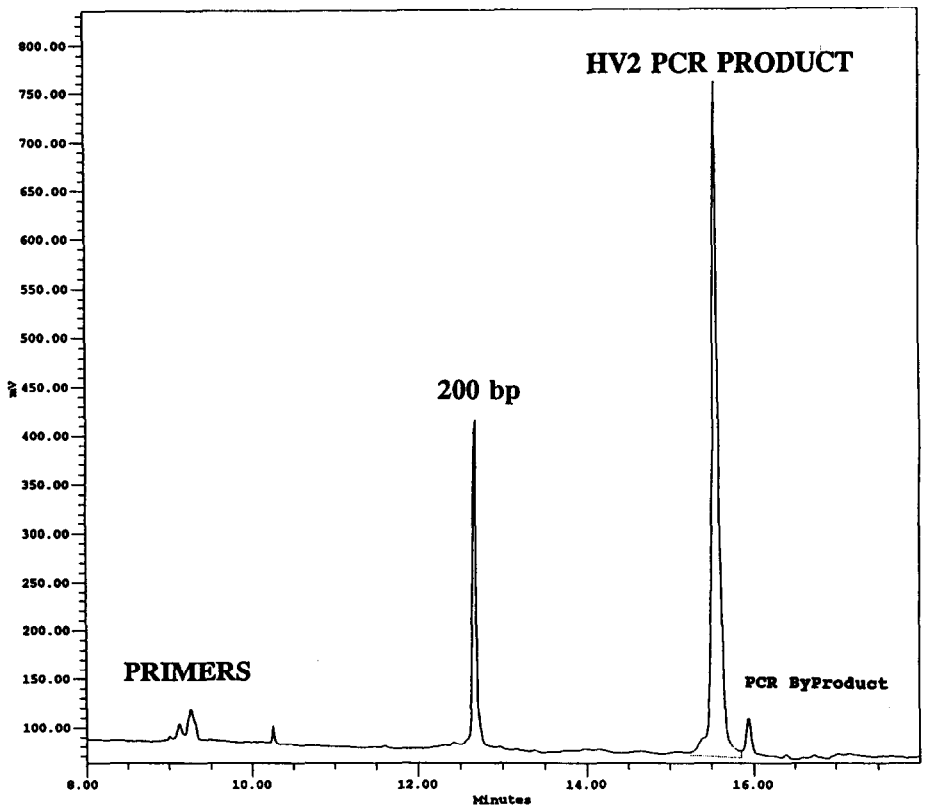

Fig. 6. A typical electropherogram of PCR product with 200-bp internal standard. Sample: 1 μl of HV2 PCR product, 49 μl of 200-bp (16 pg). Conditions as in Fig. 2, except 57 cm × 0.05 mm I.D., DB-17 column; pH 8.7; electrokinetic injection, 2 s at 5 kV; −15 kV (48 μA); 20°C.

Table 4
PCR amplified DNA quantitation methods

Parameters	Slab gel [5,6]	Slot blot [7]	Fluor spec [8]	CE–LIF
Sensitivity (MDQ)	1–5 ng (ethidium)	150 pg	1 ng	700 pg
Precision	N/A	N/A	<1% R.S.D.	3–8% R.S.D.
Resolution	1 bp	N/A	N/A	4–5 bp
Sample required	4–5 μl	5 μl	5 μl	1 μl
Analytical time (per sample)	2+ h[a]	1.5 h[a]	30+ min[a]	10–30 min
Instrument cost	low	low	moderate	high
Linear range	depends on stain	0.1–10 ng (film)	1–200 ng	0.7–250 pg
Currently automated	no	no	no	yes

[a] Multiple samples can be run at the same time.

tests. Currently, 5 μl of a 25-μl sample are used for slab-gel analysis as a quality check, and an additional 5 μl of the sample may be used to quantitate the sample by fluorescent spectrophotometric analysis. The CE-LIF method introduced in this paper furnishes the same information with consumption if only 1 μl of the PCR sample. Characterization of a PCR product can now be performed by capillary electrophoresis with appropriate resolution, sensitivity, and precision.

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