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Direct quantification of polymerase chain reaction fragments using field-amplified sample injection in capillary zone electrophoresis for gene dosage estimation

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Abstract

To assess gene dosages for clinical application, especially for prognostication of cancer, we developed a direct quantification method for polymerase chain reaction products. We report on an application of field amplified sample injection (FASI) to capillary zone electrophoresis which allows the quantification of PCR products without sample preparation. Using an external standard and UV detection for the quantification of DNA, a low coefficient of variation has been obtained. Overall, the described method provides a fast and easy tool for PCR product quantification in clinical laboratories.

Keywords: Polymerase chain reaction products; DNA

1. Introduction

Capillary electrophoresis (CE) is an appropriate method for separating and quantifying biomolecules. Several separation methods are available for CE, among others capillary zone electrophoresis (CZE). In CZE, molecules are separated by their mass:charge ratio [1,2], a feature which cannot be applied to DNA [3]. However, modifications of CZE using separation buffers that contain stiff, extended polymer molecules (e.g., methyl cellulose or hydroxyethyl cellulose) in low concentrations allow separation of DNA fragments with high resolution [4–7]. Use of the correct injection method is essential if such high resolution is to be obtained. Commonly used electrokinetical injection methods are diminished by high salt concentration often present in biomedical samples and require sample purification [8].

We have developed a method for high-resolution separation and reliable quantification of DNA fragments in the presence of high salt concentrations. A new DNA quantification method is described based on modified CZE with an improved electrokinetical injection method called field amplified sample injection (FASI) [9,10] for direct evaluation and quantification of PCR products. The modified FASI we applied consists of an additional pressure injection of water before high electric field injection of the sample. As a result, the local electrical field

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during sample application is increased which leads to higher injection efficiency and peak sharpening, and allows also a high-voltage injection over several seconds without sample overloading.

Recently, we developed a method of quantitative multiplex PCR (differential PCR, dPCR) for assessment of *erb*B-1, *erb*B-2 and *erb*B-3 gene dosage [11,12]. Many other applications of multiplex PCR for gene dosage estimation in diagnostics are reported in the literature, e.g., detection of trisomies or loss of heterozygosity [13,14]. Our CE method yields highly reliable quantitative results from PCR samples directly out of the reaction tube and allows gene dosage estimation to be used in routine diagnostics. The quantification method with FASI may be applied with any CE system using UV detection and uses only quickly prepared, nontoxic reagents.

2. Experimental

CE was performed on the capillary electrophoretic system I (with the evaluation software: PEAKNET 1.1, Dionex, Idstein, Germany) and was compared to the performance on Biofocus 3000 (evaluation software: BIOFOCUS V 5.0, Biorad, Munich, Germany) and the crystal CE system with BUTLER (Unicam, Kassel, Germany). Methylsilyl-coated (coating diameter 0.5 μm) fused-silica capillaries (360 μm O.D, ×100 μm I.D.) [OV-1 (CS Chromatographie Service, Langerwehe, Germany)] of 45-cm effective length were used. On column detection was performed by UV absorption at 260 nm. Samples were separated in an electric field of −250 V/cm (detector at the anode) in hydroxyethyl cellulose entanglement buffer: 10 g hydroxyethyl cellulose (HEC, M_r=~350 000, Fluka, Neu-Ulm, Germany) in 1 l TBE buffer (45 m*M* Tris and boric acid, 1 m*M* EDTA, pH 8.3). Particles and air bubbles were eliminated by centrifugation at 1500 *g* for 2 min. The HEC buffer was replaced after each run to maintain the same separation conditions.

Samples were injected without any purification or concentration procedures. Different injection potentials (−250, −300, −336 and −360 V/cm for 20 s) were tested to find the optimal one. FASI was tested by applying 1, 2 and 3 s double distilled water (DDW) at 1 bar with an electrokinetical injection of −336 V/cm for 20 s. dPCR was performed as in [11,14].

Restriction fragment standards, ϕX174 *Hae*III, ϕX174 *Hin*fI, nucleotides and primers were injected from a PCR reagent mixture as used for dPCR, for the identification of dPCR products. Quantification of dPCR products was achieved using dilutions of 10, 15, 18 and 20 μl of the fragment standard ϕX174 *Hae*III (1 mg/ml). A standard curve was achieved by plotting all concentrations of the 72, 118, 194 and 234 bp fragments versus molecular weight marker (MWM) peak areas. The injection was carried out by FASI.

Gel electrophoresis was done with 4% NuSieve (FMC, Rockland, ME, USA) agarose gels and 0.5x TBE. The gels were stained after electrophoresis with ethidium bromide. The pictures of the gels were evaluated with SCANPACK 1.0 (Biometra, Goettingen, Germany).

The coefficients of variation (C.V.s) were determined by ten independent measurements of a pool of *erb*B-1 differential PCR products.

3. Results and discussion

DNA fragments were separated according to ascending base pair numbers using HEC buffer and a methylsilyl-coated column (OV-1) which effectively eliminates electroendoosmosis. The effectiveness of this system was confirmed by good resolution separation of DNA (MWM) ϕX174 *Hin*fI (Fig. 1)and ϕX174 *Hae*III (data not shown). The variation of injection conditions showed an optimal injection with 3 s DDW at 1 bar and an electrical field of −336 V/cm. A comparison of separations of the same sample injected with conventional electrokinetic injection (Fig. 2) and electrokinetic injection by FASI (Fig. 3) demonstrated the advantage of FASI. The first four peaks represent nucleotides followed by the Triton X-100 peak as part of the PCR reagent mixture and the peaks of the four primers (23–25 bp). Primer dimers were detected as a broad multiclimax peak migrating between the primers and the PCR products. The peaks of the PCR products are baseline resolved with a fivefold higher sensitivity (signal-to-noise) using FASI (Figs. 2 and 3). Replacing the HEC buffer without carryover at a low filling

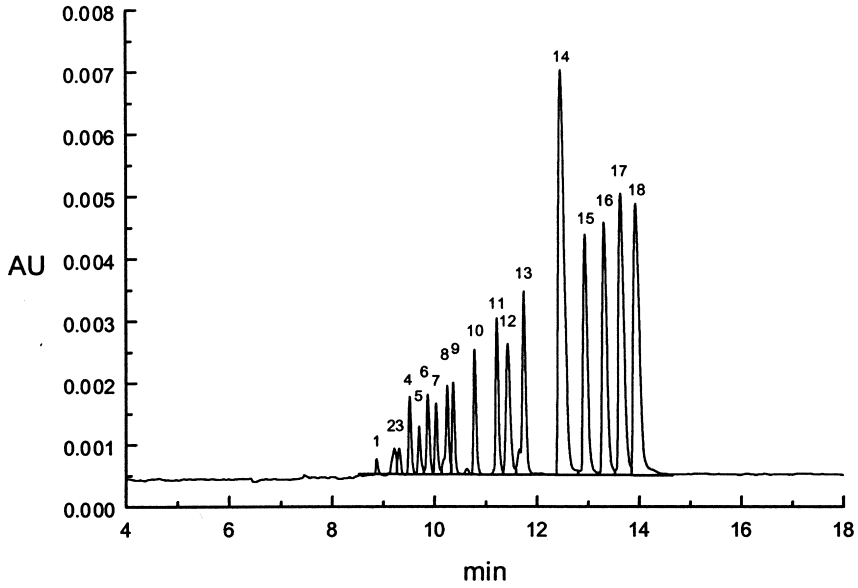

Fig. 1. Separation of DNA molecular weight marker (MWM) indicating the separation efficiency of hydroxyethyl cellulose entanglement buffer (HEC buffer see Section 2) at an electric field of −300 V/cm. *Hin*f I MWM (0.2 mg/ml) fragments: 1: 24 bp, 2: 40, 3: 42 bp, 4: 48 bp, 5: 66 bp, 6: 82 bp, 7: 100 bp, 8: 140 bp, 9: 151 bp, 10: 200 bp, 11: 249 bp, 12: 311 bp, 13: 413+417+427 bp, 14: 500 bp, 15: 553 bp, 16: 713 bp, 17: 726 bp.

pressure of only 1 bar maintains a high reproducibility of migration times and peak shape. The maximum difference in times of migration of the same PCR products from run to run was 0.3 min (±2%).

The calibration curve for quantification obtained by plotting all concentrations of the 72, 118, 194, 234 bp fragments of the differently diluted ϕX174 *Hae*III versus MWM peak areas (Fig. 4) indicates a

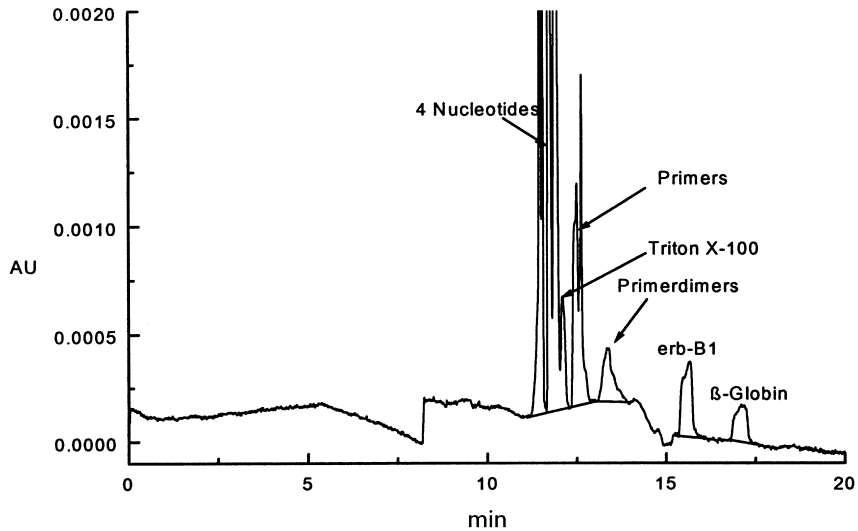

Fig. 2. Conventional electrokinetic injection at an electric field of −336 V/cm for 20 s.

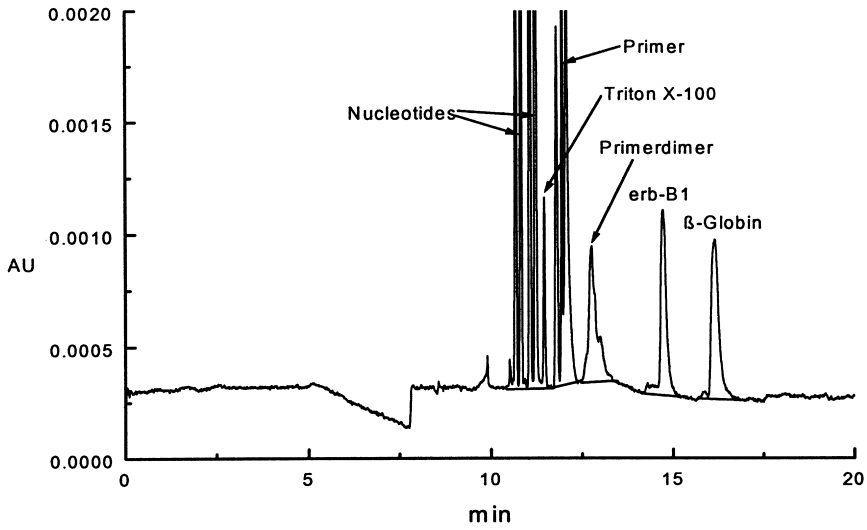

Fig. 3. Separation of the two dPCR products from *erb*B-1 and HBB at an electric field of −250 V/cm. Electrokinetic injection by FASI for 20 s (3 s, 1 bar water pressure at an electric field of −336 V/cm).

high correlation of total concentration to peak area (r=0.992). This and a C.V. value of this whole quantification method of 7.4% for a 144 bp fragment and 4.0% for a 252 bp fragment shows that this method is superior to quantification methods with a sole electrokinetic injection [15] and comparable to

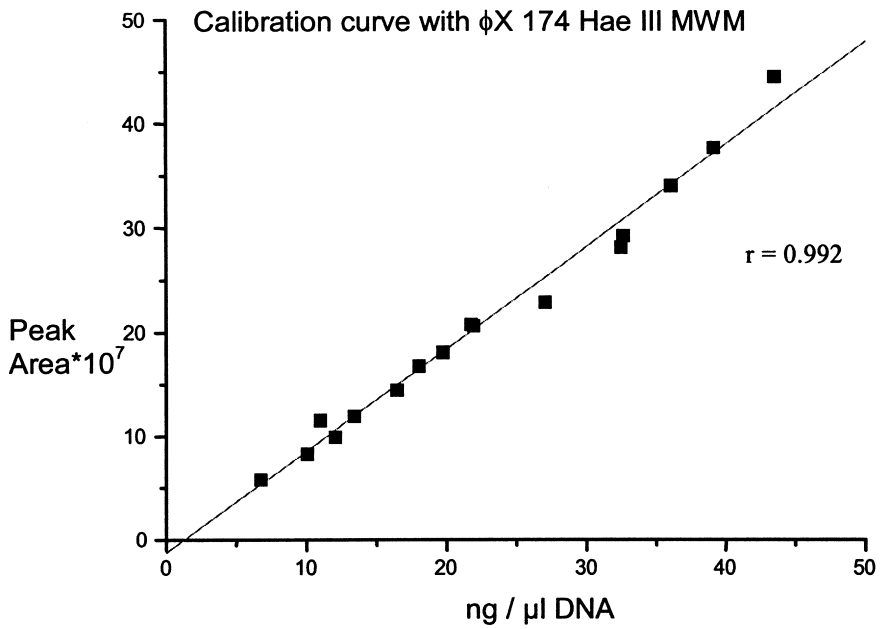

Fig. 4. Sixteen-point-calibration curve from ϕX174 *Hae*III DNA for quantification of dPCR products by CZE. Concentration of ϕX174 *Hae*III DNA in ng/μl on the *x*-axis (standard concentrations: 7–45 ng/μl), integral of extinction ($0.5\cdot10^8$–$4.5\cdot10^8$) on the *y*-axis (r=0.992).

FASI methods with prior purification steps [15] which allows the direct quantification of DNA fragments. The limit of detection for dPCR products with FASI was lower than 7 ng/μl for different DNA MWM using UV absorbance.

The reliability of the quantification method was tested by applying it to gene dosage estimation. Gene dosage measurements of *erb*B oncogenes by dPCR is comprised of coamplification of the single-copy human betaglobin (HBB) gene and the *erb*B oncogene in the same reaction tube. The gene dosage is reflected by the ratio of peak integrals of the electrophoretically separated oncogeneś (*erb*B-1, 144 bp; *erb*B-2, 132 bp; *erb*B-3, 143 bp) and the HBB reference gene's (252 bp) PCR products [10,11]. Normal diploid tissues yield a gene dosage of 1. *erb*B gene dosages below 0.4 and above 1.6 in breast, ovarian and oral cancer have an important influence on the course of disease [10,11,14]. Thus, high reproducibility and accuracy for the determination of the gene dosages is required.

Table 1 shows gene dosages for *erb*B-1 and *erb*B-2 from three tumour cell lines (A-431, MDA-MB-468 and SK-BR-3), eight patients and lymphocytes from normal subjects. The values determined by gel electrophoresis are compared to those obtained by the reported CZE method. The comparison shows that gene dosages determined by the CZE method are confirmed by using slab gel electrophoresis but show a higher precision with a C.V. of 7.4% in comparison to a C.V. of 9.9% determined by slab gel electrophoresis [16].

In addition, no significant differences were obtained by applying the method to three different automated CE systems (capillary electrophoresis system I, Dionex; Biofocus 3000, Bio-Rad; Crystal capillary electrophoresis system, Unicam). The need for a low filling pressure of only 1 bar and UV detection allows application to most commercially available CE systems.

We conclude that the method reported here is reliable for applications which require routine determination of accurate PCR product quantity without sample preparation. It has been applied for more than 400 separate runs so far to determine *erb*B-1, *erb*B-2 and *erb*B-3 gene dosages of different samples. The electrophoretic runs remained stable with regard to baseline, peak shape and separation time (±0.3 min). The method is inexpensive and uses only nontoxic, quickly prepared reagents and might also prove to be a helpful tool for clinical gene dosage estimations, especially for assessing the prognosis of different tumors [14,17–19].

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Table 1
Gene dosage (concentration of oncogene's PCR product divided by concentration of HBB's PCR product) by CZE with FASI or by gel electrophoresis, compared to gene dosage references for three tumor cell lines, lymphocytes from normal subjects and tumor tissue from eight breast cancer patients

Samples	Oncogene	Gene dosage by CZE	Gene dosage by gel electrophoresis	Gene dosage references
A431	*erb*B-1	10.2	6.4	8–20 [20,21]
MDA-MB-468	*erb*B-1	10.3	11	8–32 [22,23]
SKBR-3	*erb*B-2	4.3	3	4–8 [22]
Lymphocytes	*erb*B-1	1.1	1.3	
Lymphocytes	*erb*B-2	0.8	0.6	
Patient 1	*erb*B-1	6.6	9.8	
Patient 2	*erb*B-2	18.5	28.6	
Patient 3	*erb*B-1	6.7	5.8	
Patient 4	*erb*B-2	3.4	2.6	
Patient 5	*erb*B-1	2.1	2.5	
Patient 6	*erb*B-2	7.8	5.2	
Patient 7	*erb*B-1	0.8	1.1	
Patient 8	*erb*B-2	1.8	1.6	

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