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Capillary electrophoresis with laser-induced fluorescence detection for fast and reliable apolipoprotein E genotyping

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Abstract

The use of capillary electrophoresis (CE) with laser-induced fluorescence (LIF) detection for the rapid determination of apolipoprotein E (apoE) genotypes was studied. High resolution and sensitive detection of the concerned DNA restriction fragments was achieved using CE buffers with hydroxypropylmethylcellulose (HPMC) as sieving polymer and ethidium bromide (EB) as fluorescent intercalating agent. In order to achieve adequate resolutions in short analysis times, parameters such as concentration of HPMC and EB, separation voltage, and length and coating of the capillary were evaluated. Using a separation buffer with 0.8% (w/w) HPMC and 7 μM EB, characteristic DNA-fragment profiles could be obtained for all common apoE genotypes at an overall rate of ten samples per hour. The method allows direct injection of untreated PCR samples and the use of standard fused-silica capillaries which are effectively coated following a short, one-step rinse procedure. With a simple computerized algorithm based on migration-time ratios for pattern assignment, highly reliable apoE genotyping was achieved. Overall, in terms of speed, ease of use and objectivity the presented method provides a significant improvement over previously reported CE-based procedures for apoE genotyping.

Keywords: Genotyping; Apolipoprotein E

1. Introduction

Apoliporotein E (apoE) is an essential constituent of several plasma lipoproteins, and plays an important role in lipoprotein metabolism by serving as a ligand for apoE receptors [1]. Human apoE is a product of a polymorphic gene with three common alleles (ε2, ε3, ε4) coding for three isoforms (E2, E3, E4) [2]. These apoE isoforms differ by a cysteine–arginine interchange that occurs at one or both of two locations (residues 112 and 158) of the polypeptide chain [3], which are in the receptor-binding domain. ApoE alleles are codominantly inherited, giving rise to six genotypes: three homozygotes (ε2/ε2, ε3/ε3, ε4/ε4) and three heterozygotes (ε2/ε3, ε2/ε4, ε3/ε4). ApoE polymorphism is an important determinant of risk for certain diseases. For example,

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the prevalence of the ε4 allele is associated with the development of coronary artery disease (CAD) and Alzheimer disease [4,5], while over 90% of patients with familial dysbetalipoprotienaemia are genotype ε2/ε2 [6]. Considering the clinical significance of apoE polymorphism, the availability of reliable analytical methods for accurate and rapid apoE genotyping is of importance [7,8].

By employing the polymerase chain reaction (PCR) for amplification of the appropriate part of the apoE gene, various methods have been developed to demonstrate the underlying point mutations of common apoE genotypes [7,9–13]. To date, apoE genotyping is mainly performed by PCR followed by digestion with restriction enzymes and restriction-fragment-length-polymorphism (RFLP) analysis, according to a procedure originally described by Hixson and Vernier [14]. Separation of the resulting double-stranded (ds) DNA fragments is carried out by electrophoresis on agarose or polyacrylamide gels analyzing several samples simultaneously. Slab-gel electrophoresis, however, is often still quite time consuming, labor intensive, hard to automate, and requires significant amounts of sample. Besides, data acquisition has to take place after the separation step in an off-line fashion, and, moreover, small DNA fragments usually are difficult to detect giving rise to faint and ambiguous bands. In order to circumvent these problems, more recently, capillary electrophoresis (CE) has been utilized in RFLP analysis. With CE, high-resolution, size-based separations of DNA molecules can be obtained by adding soluble sieving-polymers to the run buffer [15]. With respect to conventional electrophoresis, CE potentially offers gains in speed, separation efficiency, sensitivity and degree of automation. Furthermore, on-capillary detection provides data in real time and thus the possibility to type the obtained restriction-fragment profile directly after CE analysis.

Until now three papers have been published on the potential of CE for RFLP-based genotyping of apoE [16–18], but the presented methods still comprise some serious disadvantages. For instance, in two of the methods [16,17] an extensive and time-consuming sample preparation involving purification, desalting and concentration is required in order to allow UV absorbance detection and/or to avoid interfering peaks. In the third method [18], in which CE is combined with laser-induced fluorescence (LIF) detection, sample desalting is not needed, but the CE procedure per apoE genotype exceeds 20 min, leading to prolonged total analysis times. In all three methods, covalently-coated capillaries (from commercial sources) are used. Unfortunately, this type of capillary—which is rather costly—often gives rise to problems such as capillary fouling, coating inhomogeneity and coating instability, resulting in seriously reduced DNA resolution and limited lifetimes of the capillaries. Another shortcoming of the reported studies is that no attention is paid to the objective interpretation of the obtained DNA-fragment profiles, that is, it is not clear how the observed migration times lead to a specific genotype. Most probably, visual recognition of the peak pattern plays a dominant role in these processes which, therefore, are rather subjective.

This paper reports on the optimization of CE–LIF for RFLP-based genotyping of apoE, with emphasis on the enhancement of resolution and reduction of analysis time. For this purpose, parameters such as concentration of sieving polymer and fluorescent intercalating dye, capillary length, and separation voltage have been studied. Special attention is also devoted to the quick and effective coating of fused-silica capillaries and to computerized data-handling for objective pattern interpretation. It will be shown that for each apoE genotype a characteristic DNA fragment profile can be obtained in less than 6 min using common fused-silica capillaries. The presented method allows direct injection of untreated PCR samples, requires no internal standards, and yields accurate data for reliable computer-based apoE genotyping.

2. Experimental

2.1. Chemicals

Tris-(hydroxymethyl)aminomethane (Tris), EDTA disodium salt, ethidium bromide (EB) and pBR322 DNA *Hae*III digest size marker were from Sigma (St. Louis, MO, USA). The pBR322 digest comprises 22 dsDNA fragments with a length ranging from 8 to 587 base pairs (bp). Hydroxypropylmethylcellulose (HPMC) for CE with a viscosity of

≈4000 cP for a 2% aqueous solution at 25 °C was also obtained from Sigma. Boric acid was from Merck (Darmstadt, Germany), thiazole orange from Aldrich (Gillingham, Dorset, UK) and TO-PRO-1 from Molecular Probes (Eugene, OR, USA). All chemicals were p.a. grade or better. CE–SDS protein run buffer (NonGelSievingBuffer) was from Bio-Rad (Hercules, CA, USA). Deionized and purified water was obtained from an Elga pure water system (Salm en Kipp, Breukelen, The Netherlands).

2.2. CE system

CE separations were performed on a P/ACE 5500 system of Beckman (Fullerton, CA, USA) equipped with a LIF detection system consisting of a Beckman laser module 488 (3 mW air-cooled argon-ion laser operating at 488 nm), an optical fiber for guidance of the excitation light to the CE system, a Beckman P/ACE LIF detector and a LIF-dedicated capillary cartridge. Prior to detection by a photomultiplier the emission light was passed through both a 488-nm notch filter and a long-pass filter (cut-on wavelength, 530 nm) of Oriel (Stratford, CT, USA).

Separation buffers were prepared by dissolving 0.5–0.8% (w/w) HPMC in TBE buffer using overnight magnetic stirring, and were stored at room temperature. The TBE buffer (pH 8.4) consisted of 89 m*M* Tris, 89 m*M* boric acid and 2 m*M* EDTA, and was filtered through a 0.45-μm membrane filter (Schleicher and Schuell, Dassel, Germany) before use. Fluorescent intercalating dye (EB, TO or TO-PRO-1) was added to the separation buffer at the desired concentration (1–7 μ*M*).

CElect-N capillaries (37 or 57 cm×75 μm I.D.) with a neutral hydrophilic coating from Supelco (Zwijndrecht, The Netherlands) and uncoated fused-silica capillaries (27 or 37 cm×50 μm I.D.) from Composite Metal Services (The Chase, Hallow, UK) were used. Before use bare fused-silica capillaries were rinsed at 20 p.s.i. with water (5 min), 1 *M* sodium hydroxide (5 min) and TBE buffer (2 min), and subsequently coated by flushing with CE–SDS protein run buffer (2 min) (1 p.s.i.=6984.76 Pa). To regenerate the coating, the last flush was repeated after approximately 20 analyses.

Electrophoresis was carried out in the reversed polarity mode (negative potential at the capillary inlet) using a typical voltage of −10 or −15 kV. The capillary was commonly thermostated at 25 °C and flushed with separation buffer (4 min at 20 p.s.i.) prior to analysis. DNA samples were electrokinetically injected at −10 kV for 1–10 s. To avoid injection-related artefacts and enhance sample loading, an electrokinetic 'preinjection' of water (3 s at −5 kV) was performed prior to sample introduction [20]. Data were acquired and analyzed using Beckman P/ACE STATION software version 1.21.

Algorithms for the assignment of apoE genotypes were developed with EXCEL97 SR-2 software (Microsoft, Redmond, WA, USA). The assignment of a peak profile was based on the positions of its three last-migrating peaks. The migration times were entered in descending order into the computer program and compared with the reference values for the apoE fragments as obtained by analyzing an ε2/ε4-calibration sample. The algorithm was programmed to accept a deviation of a certain percentage (0.5, 1, 2 or 3%) from the reference values. Based on the matching of measured and reference values, the algorithm assigned the pattern to a specified apoE genotype. Since the analyzed samples originated from previously typed individuals, we could check whether the specified genotype was correct or not. When the measured migration times matched none of the genotypes, the algorithm indicated 'no type'. When more than one genotype was matching, 'several types' were indicated.

2.3. DNA isolation, PCR amplification and HhAI digestion

DNA was extracted and purified from 200 μl of whole blood using a QIAamp blood kit (Qiagen, Chatsworth, CA, USA) according to the manufacturer's instructions. The blood was drawn from a selected group of consenting individuals who represent all the common six phenotypes of apoE and had been previously typed by a PCR–RFLP method employing agarose gel electrophoresis [19]. Each isolated DNA sample was collected in 100 μl of water and stored at 4 °C.

For each DNA sample a 267-base target sequence of the apoE gene was amplified by PCR in a DNA thermal cycler (Perkin-Elmer, Norwalk, CT, USA) according to Reymer et al. [19] with minor modi-

Table 1
Characteristic *Hha*I restriction fragments for the six common apoE genotypes

	Fragment length (base pairs)[a]					
Genotype						
ε2/ε2	*38*				*91*	*104*
ε3/ε3	38	*48*	*56*		*91*	
ε4/ε4	38	*48*	*56*	*72*		
ε2/ε3	38	48	*56*		*91*	*104*
ε2/ε4	38	48	56	*72*	*91*	*104*
ε3/ε4	38	48	*56*	*72*	*91*	

[a] Specific fragments used for genotyping are italicised.

fications. This method employs two oligonucleotide primers (BMTC facility, University Hospital Groningen, The Netherlands) with the following sequences: 5′-TAAGCTTGGCACGCTGTCCAAGGA-3′ (F6) and 5′-ATAAATATAAAATATAATAAATAACAGAATTCGCCCCGGCCTGGTACAC-3′ (F4e). Primer F4e was obtained by elongating the F4 primer, as described by Hixson and Vernier [14], with a 23-nucleotide AT tail.

The PCR samples (39 μl each) were digested with restriction endonuclease *Hha*I (Pharmacia Biotech, Uppsala, Sweden) by adding 0.5 μl of a 1000-U/ml solution and incubating for 3 h at 37 °C. *Hha*I recognizes the sequence 5′-GCGC-3′ and consequently leads to a unique combination of characteristic restriction fragments for each of the six common apoE genotypes (Table 1). The resulting samples were stored at 4 °C.

3. Results and discussion

3.1. Method optimization

3.1.1. Sieving polymer and intercalating dye

Size-dependent separation of dsDNA fragments involves the use of a proper sieving medium, and for CE many noncrosslinked polymers have been proposed which can be dissolved in buffer to obtain a replaceable gel [21]. To date, linear polyacrylamide (LPA) has gained the greatest popularity because of its excellent sieving capacity. However, LPA solutions show alkaline instability, are neurotoxic and can be very viscous requiring quite high pressures to refill capillaries [21]. We selected the polysaccharide HPMC-4000 (see Experimental) as the molecular sieve for our experiments [22]. Stable solutions [0.5–0.8% (w/w) in TBE buffer] of this type of polymer can be prepared easily and reproducibly, and have low to moderate viscosity which allows filling of 50–75 μm I.D. capillaries within 2–4 min using the standard rinse option (20 p.s.i. pressure) of the Beckman P/ACE 5500 instrument. Moreover, with HPMC-4000 sufficiently high resolutions for RFLP genotyping can be obtained in short analysis times, as will be demonstrated below.

Since UV absorbance detection appeared to be insufficiently sensitive for reliable typing of the digested PCR products, we used LIF detection for the analysis of the dsDNA fragments. On-capillary DNA labeling was achieved by adding intercalating dyes, which show strongly enhanced fluorescence when bound to DNA, to the separation buffer [23,24]. We briefly compared the fluorescent intercalators ethidium bromide (EB), thiazole orange (TO) and TO-PRO-1 at a concentration of 1 μ*M* in TBE buffer which contained 0.5% HPMC. Upon analysis of the *Hae*III digest of pBR322, which was used as DNA test mixture, similar separation performances and analysis times were observed for the three dyes. Although TO-PRO-1 and TO yielded the most favorable detection limits (respectively 35 and 3 times lower than EB), the sensitivity obtained with EB still was fully adequate for our genotyping purposes (see below). Because EB is widely available and much less expensive than TO-PRO-1 and TO, EB was used in further experiments.

We studied the effect of the HPMC and EB concentration on the DNA separation by preparing TBE buffers with 0.5, 0.6, 0.7 and 0.8% (w/w) HPMC, and 1, 3, 5 and 7 μ*M* EB (altogether 16 buffers). Since apoE genotyping requires efficient separation of relatively small fragments (Table 1), for optimization we focused on the resolution obtained for the pBR322 fragments covering the 50–130 bp region. The initial CE system comprised a Supelco CElect-N capillary (75 μm I.D.) with a total length of 57 cm. With a TBE buffer containing 0.5% HPMC and 1 μ*M* EB the DNA fragments larger than 40 bp could be resolved, with the exception of the 123/124-bp fragments (Fig. 1A). Upon raising the HPMC concentration from 0.5 to 0.8%, a consider-

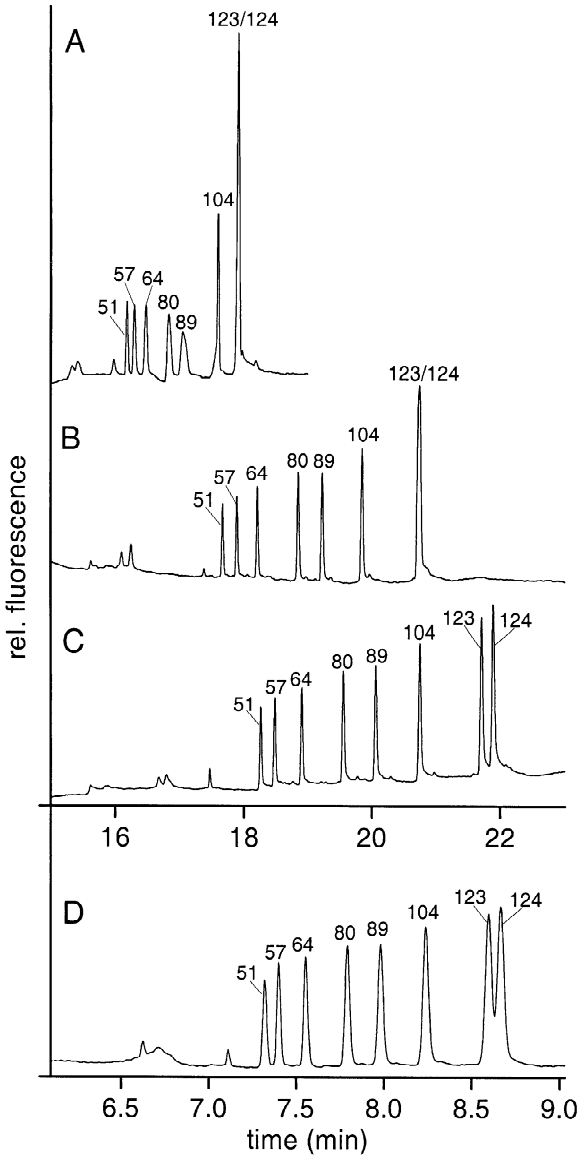

Fig. 1. CE–LIF of pBR322 fragments. Conditions: polymer and dye concentration of separation buffer, 0.5% HPMC and 1 μ*M* EB (A), 0.8% HPMC and 1 μ*M* EB (B), or 0.8% HPMC and 7 μ*M* EB (C and D); capillary, CElect-N with a total length of 57 cm (A–C) or 37 cm (D); separation voltage, −10 kV. Further conditions see Experimental. Peak annotation, DNA fragment size in bp. Note that the time scale of (D) differs from that of (A–C).

able increase of the resolution was obtained at the expense of prolonged migration times (Fig. 1B). For example, the resolution of the 80/89-bp peak pair was raised from 1.57 to 6.20. These observations are in line with earlier reports stating that for optimum separation of short DNA fragments (<300 bp) the cellulose polymer concentration should be in the 0.7–1.0% range [21,22,25,26]. HPMC concentrations above 0.8% in principle could also be used in our CE system, however, these buffers were not practical because they required prolonged rinse times to ensure complete and reproducible filling of the capillary.

Further enhancement of the resolution was achieved by increase of the EB concentration, which even resulted in baseline separation of the 123- and 124-bp fragments (Fig. 1C), indicating a single base-pair resolution in this region. The DNA intercalation of the positively charged EB causes somewhat longer migration times of DNA fragments (overall charge becomes less negative) and also induces conformational changes [27] that apparently favor selectivity among small fragments. For DNA fragments in the 50–130 bp range, the optimum separation was obtained using a TBE buffer with 0.8% HPMC and 7 μ*M* EB yielding very favorable plate numbers ranging from 1.1 to $1.5 \cdot 10^6$. Notably, high EB concentrations had an adverse effect on the peak width of fragments larger than ~300 bp, leading e.g. to overlap of the 434- and 458-bp peak (not shown).

3.1.2. Capillary length and separation voltage

With the aforementioned CE system, the achieved DNA separation is more than sufficient for apoE genotyping which demands a minimum 8-bp resolution to distinguish between the fragments of 48 and 56 bp (Table 1). In other words, the surplus of separation power can be used to gain speed of analysis. The analysis time could be decreased considerably by reducing the capillary length to 37 cm while keeping the separation voltage (−10 kV) unchanged (Fig. 1D). In this manner the migration distance is decreased while simultaneously the electric field strength is increased, leading to a 60% reduction of the migration times. At the same time the plate numbers somewhat deteriorate to values between 2.8 and $4.4 \cdot 10^5$. This loss of efficiency is mainly due to the increase of the field strength, as can be concluded from Fig. 2. Variation of the field strength between 135 and 473 V/cm (i.e. applying voltages between −5 and −17.5 kV) shows that reduction of the migration time goes along with a

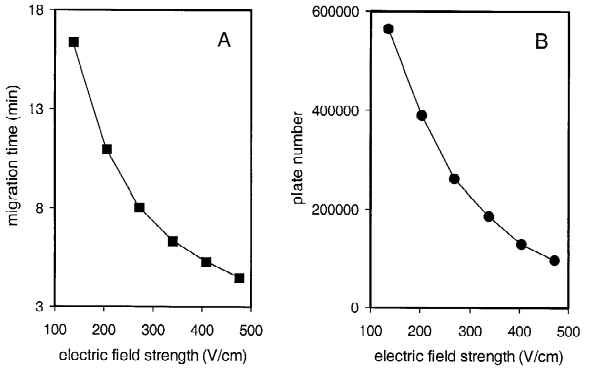

Fig. 2. Influence of the applied electric field strength on (A) the migration time (■) and (B) the plate number (●) of the 104-bp fragment during CE–LIF of the pBR322 fragment mixture. Conditions: polymer and dye concentration of separation buffer, 0.8% HPMC and 7 μ*M* EB; capillary, CElect-N (total length, 37 cm). Further conditions see Experimental.

decrease of peak efficiency and, thus, resolution. Some authors have described similar phenomena [15,28] which probably can be contributed to the effects of Joule heating and change of DNA conformation caused by higher field strengths. Further reduction of the capillary length in principle is possible, and even on 7 cm (using the short end of the capillary) we could obtain quite satisfactory DNA separations. However, with short capillary lengths the resolutions required for reliable apoE genotyping could only be obtained at significantly reduced separation voltages. These led to analysis times that were equal to or longer than the ones obtained with a capillary of 37 cm at −10 kV, that is, with a field strength of 270 V/cm. Overall, from these results it is clear that changes in the capillary length or separation voltage, for instance, to gain speed, should be applied with utmost care as they might dramatically affect the separation performance.

3.1.3. Capillary coating

Efficient size-based separations of DNA in fused-silica capillaries requires suppression of the electroosmotic flow (EOF) and analyte–wall interactions [15,29]. This can be accomplished by in situ chemical derivatization of surface silanols and covalent immobilization of a polymeric layer to the surface of the capillary. In the initial phase of this study we used commercially available capillaries with a neutral hydrophilic coating which yielded highly efficient DNA separations (see above). However, with these capillaries we sometimes observed a slow deterioration of the resolution or even a complete breakdown of performance. The involved capillaries then often showed an appreciable EOF indicating (part of) the coating was lost, and a new (costly) capillary had to be installed. Another potential method to accomplish EOF suppression is to mask the charged sites on the wall by adsorption of neutral polymers that provide a viscous layer on the capillary surface. HPMC in itself has a strong adsorptive and EOF-reducing capacity, however, we did not obtain stable and reproducible DNA separations when we used the optimum separation buffer in bare fused-silica capillaries. We found a good candidate for coating the capillary in a CE–SDS protein run buffer (NonGelSievingBuffer) of Bio-Rad which is normally used for the size-based CE separations of proteins. The buffer contains a polymer with high affinity for the capillary wall, forming stable coatings that are not removed during electrophoresis. Unfortunately, the nature of the polymer is undisclosed as the buffer is part of a commercial kit. The Bio-Rad protein buffer itself was found to be unsuitable for DNA fragment separations, but a plain preflush (2 min) of a bare fused-silica capillary with this buffer followed by normal filling with the HPMC–EB-containing TBE buffer, gave high-performance DNA separations with efficiencies ($4.0–6.8 \cdot 10^5$ plates) that were even superior to efficiencies obtained with the CElect-N capillaries (Fig. 3A). One flush was sufficient to allow for many DNA separations with satisfactory migration-time reproducibility (Fig. 3B). Commonly, after 20–30 analyses the capillary was simply regenerated by a short flush with the CE–SDS protein buffer. This process could be repeated again and again without loss of performance, extending the working life of one capillary to several months or longer. Clearly, such a coating procedure is attractive due to the ease and low costs of production and regeneration, and the possibility to use common bare fused-silica capillaries.

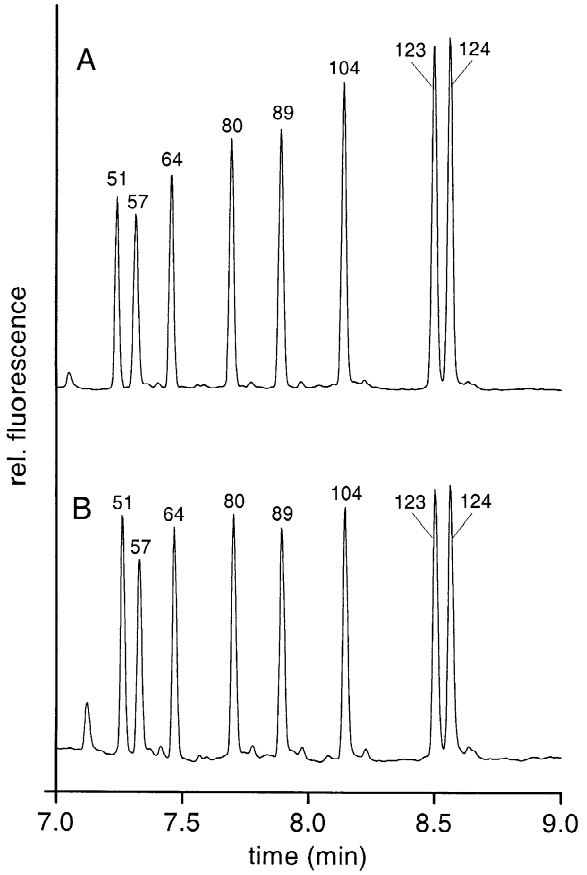

Fig. 3. CE–LIF of pBR322 fragments on capillary coated with CE–SDS protein run buffer: (A) first analysis after coating, (B) twentieth analysis after coating. Conditions: polymer and dye concentration of separation buffer, 0.8% HPMC and 7 μ*M* EB; capillary, fused-silica (total length, 37 cm); separation voltage, −10 kV. Further conditions see Experimental. Peak annotation, DNA fragment size in bp.

3.2. ApoE genotyping

3.2.1. RFLP analysis by CE–LIF

Based on the results described above, for apoE genotyping a CE system was selected which comprised a separation buffer of TBE with 0.8% HPMC and 7 μ*M* EB, and a normal fused-silica capillary (total length, 37 cm) coated with CE–SDS protein run buffer, operated at 25 °C using a separation voltage of −10 kV. With this system the PCR-amplified and *Hha*I-digested DNA of six individuals representing the common apoE genotypes were analyzed directly without any sample pretreatment. For each genotype within 8.5 min a good resolution was obtained for the apoE-characteristic DNA fragments (Table 1) revealing six unique peak patterns (Fig. 4). The untreated samples contain variable amounts of nonspecific compounds, like e.g. short *Hha*I restriction fragments and primer dimers, which migrate before or partly in overlap with the 38-bp fragment. Most importantly, for all samples no interfering peaks were observed after the 38-bp peak (i.e. the critical region for apoE genotyping) allowing appropriate and reliable RFLP analysis. On the basis of the obtained peak profiles the apoE genotypes can be easily discriminated.

Excellent fluorescence intensities were obtained for all DNA fragments. The differences in the relative intensities of the fragments are primarily caused by the fact that (i) fragments may stem from one or two alleles (Table 1), and (ii) larger fragments bind more EB and thus fluoresce stronger (see also Fig. 1). Nevertheless, it should be noted that the

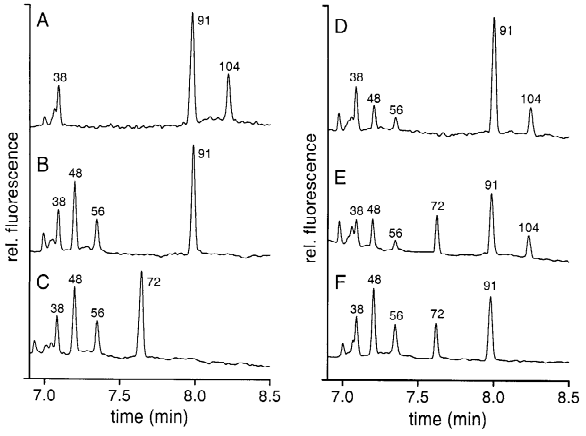

Fig. 4. RFLP analysis of the six common apoE genotypes with CE–LIF: (A) ε2/ε2, (B) ε3/ε3, (C) ε4/ε4, (D) ε2/ε3, (E) ε2/ε4, (F) ε3/ε4. Conditions: polymer and dye concentration of separation buffer, 0.8% HPMC and 7 μ*M* EB; capillary, fused-silica (total length, 37 cm) coated with CE–SDS protein run buffer; separation voltage, −10 kV; sample injection, −10 kV for 10 s. Further conditions, see Experimental section. Peak annotation, DNA fragment size in bp.

peak areas of both the 56- and 104-bp fragments are relatively low. This is most probably due to the fact that these fragments contain the AT-elongated F4 primer (see Experimental) and therefore are relatively rich of AT base pairs. The emission of EB is favored by GC base pairs in particular [30], and consequently the fluorescence intensity of the fragments of 56 and 104 bp is relatively less enhanced.

3.2.2. Computerized genotyping

In order to further study the suitability of the CE–LIF system for unambiguous apoE-genotyping, a set of 80 digested PCR-samples was analyzed over the course of several days in series of 10–20 samples. The six genotypes were practically equally represented in the sample set. Before each series the capillary was coated with the CE–SDS protein buffer and then filled with separation buffer. First an $\epsilon 2/\epsilon 4$-calibration sample, which contains all six apoE-characteristic fragments, was analyzed, followed by the sequential analysis of the samples of one series. Between every sample the capillary was refilled with fresh separation buffer by applying a 4-min rinse. With this procedure satisfactory RFLP patterns were obtained at an overall rate of 4–5 samples per hour.

Of course, the actual genotypes follow from the interpretation of the obtained CE profiles. In order to eliminate subjectivity from this process we designed a simple computerized algorithm (see Experimental). In principle, each apoE genotype can be distinguished by the three largest restriction fragments that it comprises (Table 1). Accordingly, the assignment of each peak pattern was based on the positions of its three last peaks which—assuming successful PCR and *Hha*I restriction—will always originate from apoE-specific fragments (see Fig. 4). The migration times were compared with reference values for the apoE fragments which followed from the analysis of the $\epsilon 2/\epsilon 4$-calibration sample. We tested the algorithm with various tolerances (i.e. the maximum allowed difference between measured and reference migration times). The best results were obtained with a 2% tolerance, yielding a score of 74 out of 80 (92.5%) samples typed correctly (top of Table 2). At lower tolerances a growing number of peak patterns was not recognized by the algorithm. Apparently, in this case the spread of the migration times is such that part of the peaks will not match the narrowly defined reference intervals. On the other hand, at tolerances of 2% and higher, some of the migration time reference intervals will start to overlap. This means that one peak can match more than one reference value, leading to the assignment of patterns to two or more genotypes. Notably, none of the samples was incorrectly typed. Nevertheless, the overall outcome of the automated genotyping was not fully satisfactory and had to be improved.

Table 2
Results of automated genotyping using a migration time and a migration-time-ratio based algorithm on 80 apoE profiles obtained with CE–LIF at −10 kV[a]

Tolerance (%)	One type		No type	Several types
	Correct	Incorrect		
Based on migration times				
±0.5	49	0	31	0
±1	69	0	11	0
±2	74	0	1	5
±3	52	0	0	28
Based on migration-time ratios				
±0.5	78	0	2	0
±1	80	0	0	0
±2	43	0	0	37
±3	24	0	0	56

[a] Separation buffer was replaced after each analysis.

Closer inspection of 'deviating' CE–LIF profiles showed that the measured peaks of one pattern were always shifted in the same direction and degree with respect to the reference peaks. This means that the precision could probably be enhanced by calculating relative migration times. In order to avoid the (potential) disadvantages of using an internal standard for this purpose (e.g. extra sample pretreatment, prolonged analysis times and/or interference with sample peaks), the mutual migration-time ratios for the last three peaks of the obtained apoE patterns (i.e. t_3/t_2, t_3/t_1 and t_2/t_1) were calculated. This way for each genotype a unique set of migration-time ratios was obtained. Using a similar algorithm as above, for every sample the resulting three values were then compared at various tolerances with the reference migration-time ratios as determined from the data obtained for the $\epsilon 2/\epsilon 4$-calibration sample. The results in Table 2 (bottom) clearly show that the adverse influence of the arising migration time shifts

is largely reduced by using migration-time ratios. With the algorithm set at a tolerance of 1%, all CE–LIF profiles obtained for the eighty samples were correctly assigned by the computer.

With the apoE-genotyping method described above, a large part of the total analysis time is taken up by the time needed to replace the separation buffer between each analysis. Therefore, we studied the possibility to refill the capillary only every ten analyses, i.e. using the same separation buffer for ten samples. A further gain in speed of analysis was attained by applying a separation voltage of −15 kV. Under these modified conditions, the CE–LIF system was used to sequentially analyze a set of 35 digested PCR samples. Although due to the higher voltage the plate numbers of fragment peaks were somewhat lower, still for each sample an appropriate peak profile with adequate resolution was obtained within 5.5 min. At the same time the migration-time precision deteriorated to some extent, probably because of repeated use of the separation buffer. This becomes particularly clear from Table 3 which shows that an algorithm based on absolute migration times largely fails to correctly assign the measured profiles. However, excellent results were obtained when migration-time ratios were used in the assignment procedure, yielding a score of 100% correctly assigned when a tolerance of 1% was used. In other words, the developed CE–LIF method is well suited for accurate and reliable apoE genotyping at the favorable analysis rate of ten samples per hour.

Table 3
Results of automated genotyping using a migration-time and a migration-time ratio based algorithm on 35 apoE profiles obtained with CE–LIF at −15 kV[a]

Tolerance (%)	One type		No type	Several types
	Correct	Incorrect		
Based on migration times				
±0.5	3	0	32	0
±1	5	1	29	0
±2	9	3	22	1
±3	9	5	12	9
Based on migration-time ratios				
±0.5	24	0	11	0
±1	35	0	0	0
±2	17	0	0	18
±3	8	0	0	27

[a] Separation buffer was replaced after every ten analyses.

4. Conclusions

A rapid CE–LIF method for RFLP-based genotyping of apoE was developed which requires less than 6 min per sample to accomplish adequate resolution for the distinctive DNA fragments. With the presented method interference-free peak patterns are obtained which allow unambiguous and automated assignment of all six common apoE genotypes using a computerized algorithm based on migration-time ratios. Following a quick, easy and repeatable procedure for coating, common bare fused-silica capillaries can be used in stead of covalently coated capillaries, virtually without increasing the total analysis time. The fact that no pretreatment of the PCR samples and no internal standards are needed, adds further to the swiftness and simplicity of the method, resulting in an overall gain in speed of at least a factor 3–4 when compared to a previous CE–LIF procedure for apoE genotyping [18]. On the whole, this study clearly shows that the high separation power of CE can be employed to gain speed of analysis, while LIF detection can provide the required sensitivity for the measurement of small restriction fragments. This makes CE–LIF a powerful candidate for high-throughput RFLP-based apoE genotyping.

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