Diagnosis of Duchenne/Becker muscular dystrophy and quantitative identification of carrier status by use of entangled solution capillary electrophoresis

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Use of capillary electrophoresis, a new and useful analytical tool, offers a variety of advantages for nucleic acid analyses, including rapid analysis, automation, high resolution, qualitative and quantitative results, and low consumption of both sample and reagents. We report the first example of the use of entangled solution capillary electrophoresis (ESCE) and laser-induced fluorescence detection (LIF) for separation-based diagnostics in the quantitative analysis of multiplex PCR products for determination of carrier status of Duchenne/Becker muscular dystrophy (DMD/BMD). This approach greatly improved the speed, resolution, and sensitivity of information needed for the diagnosis of DMD/BMD compared with that from conventional diagnostic methods, and is of general utility for diagnosis of genetic diseases.

INDEXING TERMS: polymerase chain reaction • heritable disorders • laser-induced fluorescence detection

Duchenne and Becker muscular dystrophy (DMD/BMD) are X-linked allelic neuromuscular defects caused by mutations in the dystrophin gene, which spans 2.5 million bases(Mb) at Xp21, that affect ~1:3500 liveborn males [1]. Partial intragenic deletion or duplications of the dystrophin gene account for as much as 72% of the DMD/BMD cases [2–7]. Newly acquired mutations account for a third of all cases [8]. The need for prenatal diagnosis of DMD/BMD and the assessment of carrier status in females at risk has provided impetus for the development of DNA-based molecular diagnostic procedures. In males, major rearrangements can be readily detected by showing absence of either an amplification product in a multiplex polymerase chain reaction (PCR) [9–12] or a hybridization signal on Southern transfer gene analysis with cDNA probes [3]. Characterization of carrier status in females as well as exon duplication in affected males is achieved through use of various quantitative approaches for gene dosage. Linkage analysis identifies carrier females without deletions by assessing restriction fragment length polymorphisms [13, 14] or intragenic polymorphic CA repeats [15]. However, a 5% meiotic recombination frequency, absence of informative meioses, de novo mutations, germline mosaicism, and uncooperative family members may complicate these studies. More technically demanding techniques such as detection of deletion junction fragments [16, 17], quantitative Southern transfer hybridization [18], and amplification of ectopic transcripts have been described for the detection of DMD/BMD [19, 20].

A comprehensive repertoire of PCR-based assays has been reported that allows quantitative gene dosage analysis [21–24]. Briefly, the defect responsible for the disease is initially detected by a PCR reaction that remains within
the logarithmic phase of amplification. The PCR product from a coamplified control exon is quantified after ethidium bromide staining [22] or densitometric scans of autoradiograms [21]. Different fluorescent labeling strategies to identify deletion carriers have also been described [23–26]. The combination of fluorescent labeling, laser-induced fluorescence (LIF) detection, and automated, quantitative assessment yields sensitivity ~1000 times greater than ethidium bromide detection with a routine UV transilluminator.

Quantitative analysis of PCR products has many applications, e.g., carrier status identification of inherited diseases among family members [27], monitoring efficiency of nucleic acid amplification [28], and quantification of specific mRNA [29]. The numerous methods developed to quantify the PCR-amplified products include DNA hybridization with chemiluminescence detection [30], slab gel electrophoresis with fluorescence detection [24], HPLC with UV detection [31], and oligodeoxynucleotide ligation assay with ELISA detection [32]. However, these methods are either labor intensive, time consuming, costly, or hazardous.

Capillary electrophoresis (CE) has been quickly established as an efficient analytical tool [33]. A typical CE system consists of two electrolyte chambers connected by a separation column, an on-line detector positioned close to the outlet of the capillary, and a high-voltage direct current power supply. The sample (~5 mL) can be introduced into the column either by electromigration or by differential pressure including applied pressure or vacuum. After the sample is loaded, high voltage is applied to the two electrodes inside the electrolyte chambers to establish an electric field across the capillary column. This can rapidly separate samples with high resolution. In many cases, the resolution limit and speed are determined by the Joule heating produced by operating at high power levels. To dissipate the heat efficiently, narrow capillaries of ~35–100 μm (i.d.) are usually used. Under such conditions, plate counts well in excess of 500 000 for individual peaks, and at times >106, are readily attainable. Compared with conventional slab gel electrophoresis, CE has advantages of automation, qualitative and quantitative results, high resolution, rapid analysis, and low sample volume/reagent consumption. The technique has been applied widely to the separation of a diverse range of compounds, including large biomolecules such as proteins and DNA. CE has been used for nucleic acid analyses in mutation detection [34–37], disease diagnosis [38–46], forensic investigations [47–49], and pharmaceutical quality control [50–52]. It has also been used for quantifying PCR-amplified products [53, 54] and ribonucleotides [55]. In some of these applications, separation has been achieved by using entangled solution CE (ESCE) in physically and chemically entangled sieving matrices based on non-cross-linked polyacrylamide, hydroxypropylmethyl cellulose (HPMC), or hydroxyethyl cellulose polymers [35–37, 46].

We report the first example of the use of ESCE and LIF detection for quantitative analysis of multiplex PCR products to identify the carrier status of subjects for DMD/BMD. This approach has great potential for increasing the speed, resolution, and sensitivity for diagnosing DMD/BMD compared with conventional methods, and serves as a practical model for the determination of carrier status for many other genetic diseases.

Materials and Methods

PCR REACTIONS
High-molecular-mass DNA was extracted from peripheral blood leukocytes by the previously described protocol [56]. Clinical samples included DNA from normal female controls, affected males with deletional forms of DMD/BMD, unrelated obligate carriers containing deletions in the dystrophin gene, and female family members at risk for being carriers. DMD/BMD genotypes were previously determined [24].

DNA concentrations were adjusted to 50 mg/L (50 ng/μL), and 100 ng of the template was used in a total reaction volume of 50 μL. PCR amplifications were for 23 and 25 cycles, in either a Perkin-Elmer (Norwalk, CT) GeneAmp System 480 or a 9600 thermal cycler, essentially as previously described [9, 10].

CAPILLARY ELECTROPHORESIS
ESCE was performed on the P/ACE system 5010 with a LIF detector (Beckman, Fullerton, CA) in the reversed polarity mode (negative potential at the injection end of the capillary column). The excitation and the emission wavelengths were 488 and 520 nm, respectively. The external temperature of the capillary column was set at 20 °C.

Before analysis, 1 μL of the multiplexed PCR products was removed from the reaction and diluted with 8 μL of deionized distilled water plus 1 μL of PCR product encompassing the β-globin gene region. Sample injections were conducted under the conditions specified in each figure legend, and the separation was performed at a field strength of 296 V/cm for 11 min in 90 mmol/L Tris-base, 90 mmol/L boric acid, and 2 mmol/L EDTA, pH 8.4, containing 5.0 mL/L glycerol and 5 g/L HPMC (H-7509; Sigma Chemical Co., St. Louis, MO). The viscosity of a 20 g/L aqueous solution of this cellulose derivative was 4000 cP at 25 °C. HPMC was dissolved in the buffer by the method recommended by Ulfelder et al. [57]. The buffer was filtered through a 4.0-μm (pore size) filter and then degassed for 15 min by sonication. Fluorescent dye was made up daily by adding YO-PRO-1 (Molecular Probes, Eugene, OR) to the above buffer to a final dilution of 1:1000.

Samples were analyzed with a surface-modified fused-silica capillary (DB-1; J&W Scientific, Folsom, CA). Postrun analysis of the data was performed with the Gold Chromatography Data System (Version 8.0; Beckman). The capillary column (27 cm × 100 μm) was conditioned
with ~10 μL of distilled water followed by ~10 μL of separation buffer and then was subjected to voltage equilibration for 30 min until a stable baseline was achieved. After each run, the column was washed with fresh separation buffer for 42 s.

**Results and Discussion**

**Separation of PCR products by CE**

The entangled separation media used in this study were the cellulose derivative HPMC and glycerol, the combination of which has both physically and chemically entangled sieving pores of different sizes and a relatively high viscosity [58]. This system has a high resolving power, provides rapid separation, and has been used for detecting mutations [35, 36]. An electropherogram of multiplex PCR products generated with the protocol reported by Beggs et al. [10] for the diagnosis of BMD is shown in Fig. 1. All nine products were well resolved to baseline in ~10 min. In comparison, the results previously reported [46] had a separation time of 50 min and produced only partial separation. An electropherogram of nine normal exons from a normal female is shown in Fig. 1A. Results from an affected male patient (Fig. 1B), in comparison, clearly shows deletion of exons 13 and 43. Separation of the mixed products from multiplexed PCR reactions by using the Chamberlain (exon 4 = 196 bp; exon 8 = 360 bp; exon 12 = 331 bp; exon 17 = 416 bp; exon 19 = 459 bp; exon 45 = 547 bp; exon 48 = 506 bp; exon 51 = 388 bp) and the Beggs protocols is shown in Fig. 2. Sixteen of 18 products were separated in 10 min—compared with 70 min reported with use of a low-viscosity polyacrylamide sieving polymer solution [46]. Each of the samples injected for CE was a dilution of the original PCR products (1 μL of sample diluted by 9 μL of water). Although the current separation system failed to resolve the 535- and 547-bp fragments (12-bp difference), it may be possible to resolve these two fragments by increasing the concentration of glycerol in the separation media [58]. LIF detection (with the intercalating fluorescent monomer dye YO-PRO-1) was ~100-fold more sensitive than the UV absorbance method used for on-line detection of the DNA molecules.

**Quantitative identification of carrier status**

**System optimization.** A battery of multiplexed PCR products was used to investigate the optimal injection conditions for quantitative PCR analysis. The test consisted of a run of four batches, with each batch containing eight identical samples. The samples were injected at 5 kV for 3, 5, 7, and 9 s. The averaged reproducibility (CV) for each injection condition is shown in Fig. 3 as both corrected CVs and uncorrected CVs. The corrected CVs were obtained by using 196-, 331-, 416-, and 547-bp fragments (for the respective injection times) as internal standards for the postrun correction of the peak area.

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**Fig. 1.** CE detection of PCR products from the DMD locus by Beggs’ multiplex DNA amplification: (A) electropherogram of a normal female with nine exons present; (B) electropherogram from the affected male patient B in whom exons 13 and 43 were deleted.

All nine products (PM = 535 bp; exon 3 = 410 bp; exon 6 = 202 bp; exon 13 = 238 bp; exon 43 = 357 bp; exon 47 = 181 bp; exon 50 = 271 bp; exon 52 = 113 bp; exon 60 = 139 bp) were separated by CE in an inverted mode and detected by using LIF. RLU, relative fluorescence units.

**Fig. 2.** CE detection of PCR products from the DMD locus by Chamberlain’s and Beggs’ combined multiplex DNA amplification.
The lowest CV was achieved for the 7-s injection at 5 kV. As indicated in Fig. 3, the most accurate postrun correction (0.59% average CV) was obtained by using the peak area of the 416-bp fragment as the internal standard. The next most accurate correction (0.76% average CV) was obtained by using the peak area standardized to the 547-bp fragment (the largest fragment). From these results and the convenience of selecting an internal control that does not overlap with any of the actual sample fragments, we chose a 756-bp PCR-amplified DNA fragment as the internal standard and an optimal injection condition of 7 kV for 5 s for the subsequent quantitative determination of carrier status of DMD/BMD.

Quantitative identification of carrier status. We used the same quantity of sample DNA for PCR reactions. Superimposed electropherograms of a normal female and a normal male are shown in Fig. 4. The peak for every product was automatically identified by its fragment size, and the peak areas were corrected with use of the 756-bp fragment as internal standard. A plot of the corrected peak areas vs the size of each fragment (Fig. 5) clearly showed that the peak areas of the amplification products for the normal female are about double those for the normal male, as expected for an X-linked gene. The superimposed electropherograms from both a normal female and a female carrier are shown in Fig. 6. The ratio of the corrected peak areas of samples A and B (Fig. 7) makes evident that sample B is from a female carrier...

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**Fig. 3.** Reproducibility (CV) of CE analysis under different injection conditions. The samples were injected at 5 kV for 3, 5, 7, or 9 s. The lowest CV was achieved with a 7-s injection at 5 kV. Three fragments spanning the range of fragment sizes (196, 331, and 547 bp) were used as internal standards for postrun correction of peak areas in investigating the accuracy of quantitative CE analysis.

**Fig. 4.** CE detection of PCR products from the DMD locus by Chamberlain’s multiplex DNA amplification. Peaks marked with Xs represent products from the normal female, and the solid-line peaks represent products from the normal male. The 756-bp fragment was the added internal control.

**Fig. 5.** Comparison of corrected peak areas (756-bp fragment internal control) for DNA fragments from exons 4, 8, 12, 17, 19, 44, 45, 48, and 51 from a normal male (●) and a normal female (■).

**Fig. 6.** Superimposed electropherograms of DNA fragments (196–547 bp) from a normal female and a female carrier. Peaks marked with Xs were from the normal female; solid-line peaks were from the female carrier who had a deletion at exon 45 (547 bp) on one X chromosome.
because of a deletion at exon 45 (547 bp) on one X chromosome, leading to a 50% reduction in the peak area for the 547-bp fragment.

In conclusion, as both Fig. 5 and Fig. 7 illustrate, accurate quantitative determination of X-linked DMD/BMD carrier status can be achieved with the PCR–quantitative CE analytical system reported here. In addition, this study indicates how the ESCE method could be used to screen for suspected carrier females in families when DNA samples from affected males are not available. This is estimated to be relevant in ~20% of families with an X-linked lethal disorder such as DMD/BMD. Unlike other methods [49, 57], prepurification or dialysis of the PCR sample is not required, greatly simplifying sample preparation for quantitative CE analysis. To achieve maximum performance for production of quantitative results, the following requirements must be met. First, the quality and quantity of the template prepared from different samples must be consistent. Second, optimization of the operational conditions must be reestablished when system features, e.g., capillary coating and dimensions, separation medium, and manufacturers, are changed. Components of the present separation system can be easily coupled for use with a capillary array electrophoresis system [59, 60], which should have a much higher sample-throughput capability than any of the currently used systems.

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