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Research Article

Genotyping of exons 1 to 20 in Duchenne muscular dystrophy by universal multiplex PCR and short-end capillary electrophoresis

One rapid CE method was established to diagnose Duchenne muscular dystrophy (DMD). DMD is a severe recessive inherited disorder frequently caused by gene deletions. Among them, exons 1–20 account for nearly 30% of occurrences. In this study, the universal multiplex PCR was used to enhance the fluorescently labeling efficiency, which was performed only by one universal fluorescent primer. After PCR, a short-end injection CE (short-end CE) speeded up the genotyping of the DMD gene. This method involved no extra purification, and was completed within 9 min. The CE conditions contained a polymer solution of 1.5% hydroxylethylcellulose in 1× TBE buffer at 6 kV for separation. This method was applied to test six DMD patients and one healthy male person. The results showed good agreement with those of multiplex ligation-dependent probe amplification. This method can be applied for clinical diagnosis of DMD disease. Accurate diagnosis of the DMD gene is the best way to prevent the disease.

Keywords:
Duchenne muscular dystrophy / Genotyping / Short-end capillary electrophoresis / Universal multiplex PCR
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1 Introduction

Duchenne muscular dystrophy (DMD) is an X-linked severe recessive disorder that can be divided into three types based on clinical characteristics and is caused by defects of the dystrophin gene [1, 2]. The dystrophin gene is the largest one among identified human genes, which contains 79 exons and spans 2.3 megabases at chromosome Xp21.2. The DMD gene produces a protein of 427 kDa [3]. The incidence of DMD is about 1 in 3500 newborn males [4–6]. Gene deletions account for about 65% [3, 4, 7], duplications about 5–10%, and small rearrangements or point mutations account for 25–30% of DMD cases [4, 8, 9]. Deletions or duplications occur particularly at exons 2 and 20 (30%) and exons 44 and 53 (70%) [4, 8]. As gene transcription errors happen in encoding proteins, the lack of the dystrophin protein results in progressive muscular weakness and dysfunction of supporting muscle fibers. There is no cure for this disease, and the only treatment is to control progression. Steroid drugs are employed in slowing progression to improve life quality.

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Abbreviations: **DHPLC**, denaturing HPLC; **DMD**, Duchenne muscular dystrophy; **HEC**, hydroxylethylcellulose; **HPMC**, hydroxypropylmethylcellulose; **IS**, internal standard; **MLPA**, multiplex ligation-dependent probe amplification; **PEO**, polyethylene oxide; **UMPCR**, universal multiplex PCR; **uni-FAM**, universal FAM-labeled primer

Accurate diagnosis of the DMD gene is the best way to prevent the disease. Several methods have been developed for detection of the DMD gene. The deletions and duplications were determined by multiplex ligation-dependent probe amplification (MLPA) [10–12], array-MLPA [13], and multiplex PCR [14–16]. Fluorescence in situ hybridization is less used in detection of deletion and duplication, but it can be used to detect small deletions [17, 18]. Small deletions, insertions, and point mutations are analyzed by denaturing HPLC (DHPLC), direct sequencing [19], single-strand conformation polymorphism (SSCP) analysis [20], detection of virtually all mutations-SSCP [21], single condition amplification/internal primer [22], denaturing gradient gel electrophoresis [23, 24], and microarray-based methodologies [6]. Among them, MLPA is the most used method for analyzing deletions or duplications of the DMD gene in clinical assays. However, MLPA is very sensitive to impurities and thus requires extra purification steps to remove impurities [4]. The protocol of MLPA DMD kit is complex and time consuming (requirement of about 24 h). We have recently reported a study based on the universal multiplex PCR (UMPCR) equipped with CE analysis that was able to detect exons 44–55 of the DMD gene [25]. However, 12 peaks in the electropherogram led to consume a lot of time in peak identifications and extend the separation time as long as 25 min. In this study, some alterations were utilized to separate exons 1–20 of the DMD gene. The method did not require the extra purification steps after PCR amplifications and only a universal

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Table 1. The concentrations and sequences of the primers, and their PCR products

Primer	Concentration (μM)	Sequence (5′→ 3′)	Product (bp)
Exon 1-F	0.012	ATAAG TGACG TACTA GCAAC GAGAG CTTGC CATGC TGGAA G	494 + 17
Exon 1-R	0.06	TCTAC CTAAT TAGTG AGCTT GTC	
Exon 2-F	0.02	ATAAG TGACG TACTA GCAAC GTCAT AATGG AAAGT TACTT TGGTT G	225 + 17
Exon 2-R	0.08	AAGAT ACACA GGTAC ATAGT CC	
Exon 3-F	0.02	ATAAG TGACG TACTA GCAAC GCCTT TTTTC ATCCG TCATC TTC	350 + 17
Exon 3-R	0.16	TGTTG TCAGT TTCTG GTCTG	
Exon 4-F	0.02	ATAAG TGACG TACTA GCAAC GAGTA GATTG TCGGT CTCTC TG	199 + 17
Exon 4-R	0.16	AGCCC TCACT CAAAC ATGAA G	
Exon 5-F	0.04	ATAAG TGACG TACTA GCAAC GGCTA AATGC AATTA CCTTC ACG	434 + 17
Exon 5-R	0.32	TTTAC CTTAT ACTTA CTCAA TG	
Exon 6-F	0.02	ATAAG TGACG TACTA GCAAC GTCTT GCTCA AGGAA TGCAT TTTC	317 + 17
Exon 6-R	0.12	AGGAC ATGAT CTGGA ACCAT AC	
Exon 7-F	0.02	ATAAG TGACG TACTA GCAAC GAGCA TGGAA GTAAA TCTCA TGG	278 + 17
Exon 7-R	0.12	TGTAG AAATG ACAAG TCTCA GATG	
Exon 8-F	0.02	ATAAG TGACG TACTA GCAAC GACTG CTCAT CTCAT TGGTC TG	458 + 17
Exon 8-R	0.08	TGTGC ACGTA ATACC TAAAA ATG	
Exon 9-F	0.02	ATAAG TGACG TACTA GCAAC GTAGT CCTTT CGGGT TACTT ATG	250 + 17
Exon 9-R	0.04	TGGAA GCAGT TCTCT GGTTT G	
Exon 10-F	0.04	ATAAG TGACG TACTA GCAAC GGGAT TTTGA CCGCT ATTTG A	410 + 17
Exon 10-R	0.24	ACGTT TTAGT TTACC TCATG	
Exon 11-F	0.02	ATAAG TGACG TACTA GCAAC GGAAC AGGAA AATTA TCAGA AG	320 + 17
Exon 11-R	0.16	ACAGT GCATC TATCT AACAT CTG	
Exon 12-F	0.02	ATAAG TGACG TACTA GCAAC GATCC TTCTC AATGT CCAAT AGATG C	460 + 17
Exon 12-R	0.1	TCAAG CCATT GCAAC AAAGA TT	
Exon 13-F	0.02	ATAAG TGACG TACTA GCAAC GATTG GCTTG GAATG GTTTT AG	410 + 17
Exon 13-R	0.1	AGCAC TTCAG CTGAT TATGA G	
Exon 14-F	0.02	ATAAG TGACG TACTA GCAAC GAGCG TACAT AGGAG ACTGA G	234 + 17
Exon 14-R	0.04	AGCTA GTTTC TCACA CATGA C	
Exon 15-F	0.02	ATAAG TGACG TACTA GCAAC GACAC GGGGG GATAT TAAAT TG	352 + 17
Exon 15-R	0.14	TCCAC CTATA GTTTT TTCCC AC	
Exon 16-F	0.02	ATAAG TGACG TACTA GCAAC GTGCA ACCCA GGCTT ATTCT GTGAT C	346 + 17
Exon 16-R	0.1	TTTAA CTAAA CACAG GGCAA AAAC	
Exon 17-F	0.02	ATAAG TGACG TACTA GCAAC GTCTG AAGGT CAATC TACCA AC	412 + 17
Exon 17-R	0.1	TTACA GGTAC CCGAG GATTC	
Exon 18-F	0.02	ATAAG TGACG TACTA GCAAC GTAAT AGAGG TGTCA GGCAG G	295 + 17
Exon 18-R	0.08	AGCAG CACAA AATGA GTAC AG	
Exon 19-F	0.02	ATAAG TGACG TACTA GCAAC GCATC TTAAG GCTTG AAAGG GCAAG	382 + 17
Exon 19-R	0.12	TCTTC CAATG AACTC AAAGT TG	
Exon 20-F	0.01	ATAAG TGACG TACTA GCAAC GTCAT TGTTG TGACG CAAGT CTG	506 + 17
Exon 20-R	0.14	CTTGG AAATT GCCAA GAAAT AC	
KRIT-1-F	0.04	ATAAG TGACG TACTA GCAAC GTTCG AATGG CTACT TCTAC CTG	558 + 17
KRIT-1-R	0.32	TAGCT AAGAC TACAG GGGTA CG	
uni-FAM[a)]	0.4	FAM-GTGAC GTACT AGCAA CG	17

a) FAM: 6-carboxy-fluorescein.

fluorescent primer was needed in UMPCR to simultaneously fluorescently label all amplicons. It is beneficial to employ UMPCR for a speedy DMD genotyping detection, due to the large number of exons in the DMD gene. Exons 44–53 and exons 2–20 almost cover all of the deletion issues in DMD. It is important to establish the method for analyzing them. Based on these features, we developed a new method to detect the regions of exons 1–20 of DMD gene. Twenty genetic fragments were divided into four groups on the basis of one internal standard, and the fragments were simultaneously amplified and fluorescently labeled. After UMPCR, the amplicons were directly analyzed by the short-end CE method. Short-end CE utilizing the length of capillary from outlet end to detector (effective length is 10 cm) was introduced to decrease the analysis time and to find several fold increase in sensitivity [26]. This simple and fast method may be feasible for serving as a diagnostic tool for detection of DMD gene in clinical analysis.

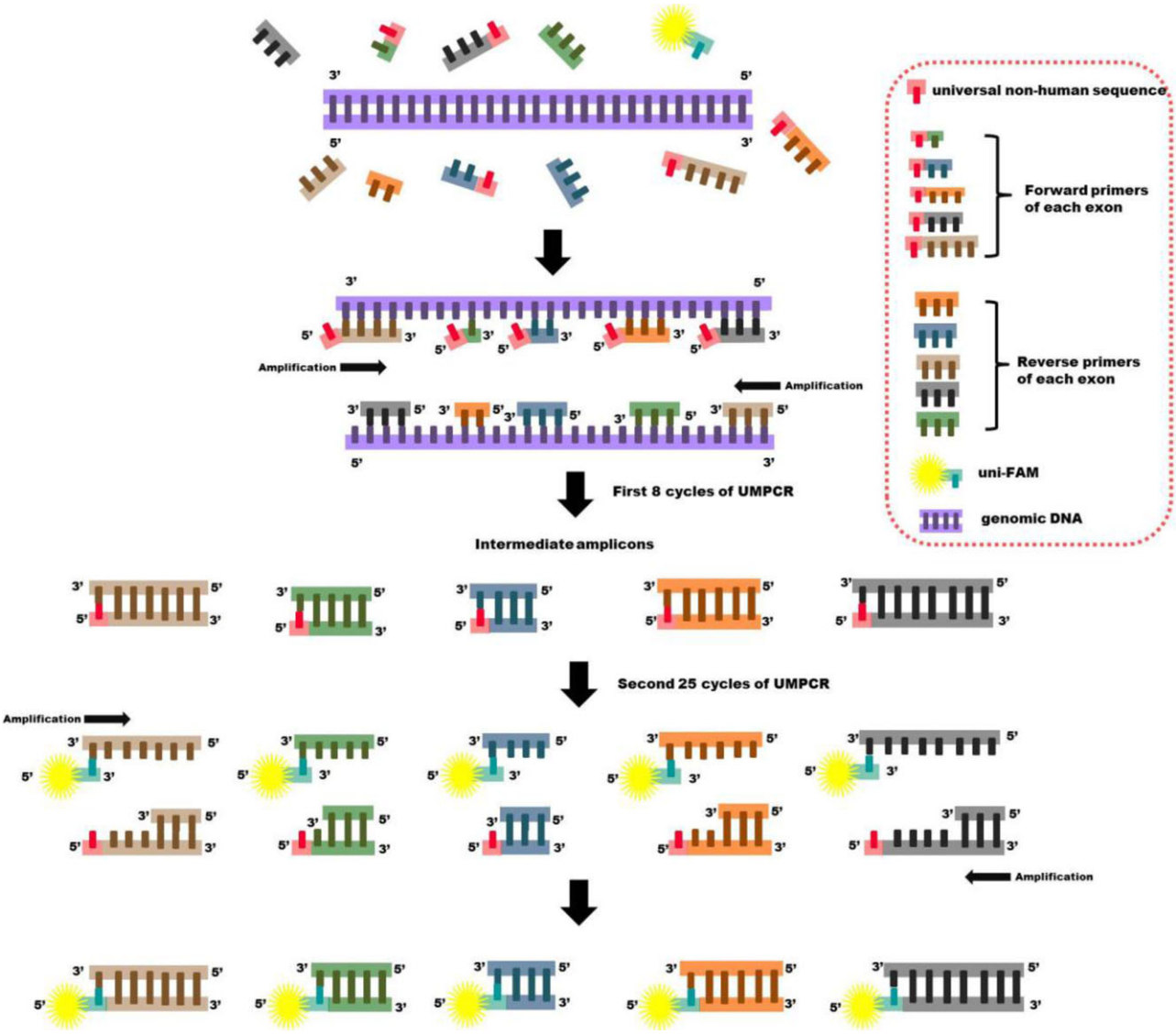

Figure 1. Schematic illustration of UMPCR. Genomic DNA was specifically amplified by the designed forward primers, which have a section of universal nonhuman sequence, and reverse primers. All of the intermediate amplicons possessed the universal sections by first eight cycles of PCR. Subsequently, the uni-FAM primer hybridized the complementary sequences of nonhuman genes and amplified them in the second 25 cycles of UMPCR. Finally, all DNA fragments were labeled with fluorophore.

2 Materials and methods

2.1 Chemicals and reagents

All primers used in this study were obtained from MDBio (Taipei, Taiwan). The e2TAK DNA polymerase, dNTP and 5× PCR buffer were purchased from Takara Bio (Ohtsu, Japan). The 5× TBE buffer was purchased from Protech Technology Enterprise (Taipei, Taiwan), and was diluted to 1× with deionized water. Polyethylene oxide (PEO) (MW 8 000 000) and hydroxypropylmethylcellulose (HPMC) (2% in water, viscosity of 15 000 cps) were purchased from Sigma-Aldrich (St. Louis, MO). Hydroxylethylcellulose (HEC) (1% in water, viscosity of about 145 mPa·s) was purchased from Fluka (Buchs, Switzerland). Methanol was obtained from Merck (Darmstadt, Germany). Deionized water was purified with a Milli-Q system from Millipore (Bedford, MA, USA). Quick-gDNA MiniPrep-D3024 was a DNA purification kit of Zymo Research (Irvine, USA).

2.2 Instrumentation

This experiment was performed on a Beckman P/ACE MDQ system (Fullerton, CA, USA) equipped with a laser-induced fluorescent detector. The wavelengths of excitation and emission were 488 and 520 nm, respectively. A DB-17-coated capillary was used, which was purchased from Agilent Technologies (Palo Alto, CA). UMPCR was performed on Biometra thermocycler (Goettingen, Germany).

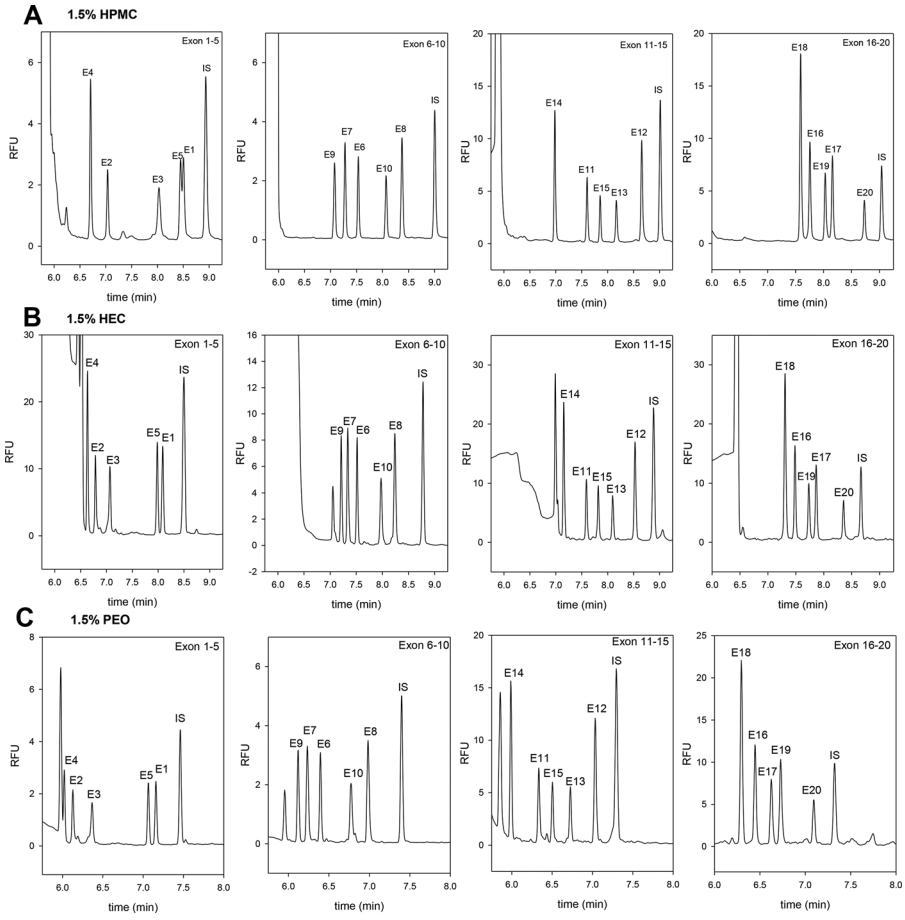

Figure 2. Effects of different types of polymer solutions on the separation of exons 1–20 of DMD gene by using UMPCR equipped with short-end CE method. (A) 1.5% HPMC, (B) 1.5% HEC, and (C) 1.5% PEO in 1× TBE buffer. The DNA sample is obtained from a healthy male volunteer. Other CE conditions were as follows: sample loading, 10 kV for 20 s; separation voltage, 6 kV; capillary temperature, 25°C; DB-17 coated capillary, 10.0 cm (effective length) × 100 μm id. Peaks identification: IS: KRIT-1, E1–E20 represent exons 1–20, respectively.

2.3 Preparation of genomic DNA samples

The wild-type DNA sample was collected from a healthy male volunteer. DNA samples of DMD patients were obtained from Kaohsiung Medical University Hospital (KMUH). The genetic DNAs were obtained from whole blood samples by using DNA purification kits. The ethics approval for this study was obtained from the Institutional Review Board at KMUH, where participants were recruited and experiments were conducted. Written informed consents were obtained from the participants.

2.4 UMPCR conditions

The UMPCR was carried out by using 50 ng of extracted human genomic DNA as the template. The total 25-μL reaction volume contained 50 ng of genomic DNA, 2.5 mM dNTP, 2.5 U of e2TAK DNA polymerase, and identical concentration of each primer (Table 1) in 1× PCR buffer. The PCR was performed with the following steps: 95°C for 10 min, followed by eight cycles of denaturation at 95°C for 30 s, annealing at 60°C for 2 min, extension at 72°C for 1 min, then 25 cycles of denaturation at 95°C for 30 s, annealing at 52°C for 20 s, extension at 72°C for 1 min, and a final extension step at 72°C for 10 min. After the PCR, the amplicons were analyzed by the CE method.

2.5 Short-end CE method

The separation was performed on a DB-17-coated capillary (total length, 40 cm; short end, 10 cm; id, 100 μm). The new capillary was preconditioned by methanol (40 psi for 10 min) and deionized water (40 psi for 10 min) sequentially. The gel solution consisted of 1.5% HEC in 1× TBE buffer. Before the runs, the gel solution was injected into the capillary at 45 psi for 60 min. Between runs, the capillary was rinsed with the gel solution at 45 psi for 10 min. The PCR samples were diluted with deionized water in the ratio of 1:4. The sample was injected at 10 kV for 20 s. The separation voltage was set at 6 kV. The cartridge temperature was set at room temperature (25°C).

3 Results and discussion

3.1 UMPCR

This study combined UMPCR and short-end injection CE for simultaneously genotyping DMD exons 1–20. In UMPCR,

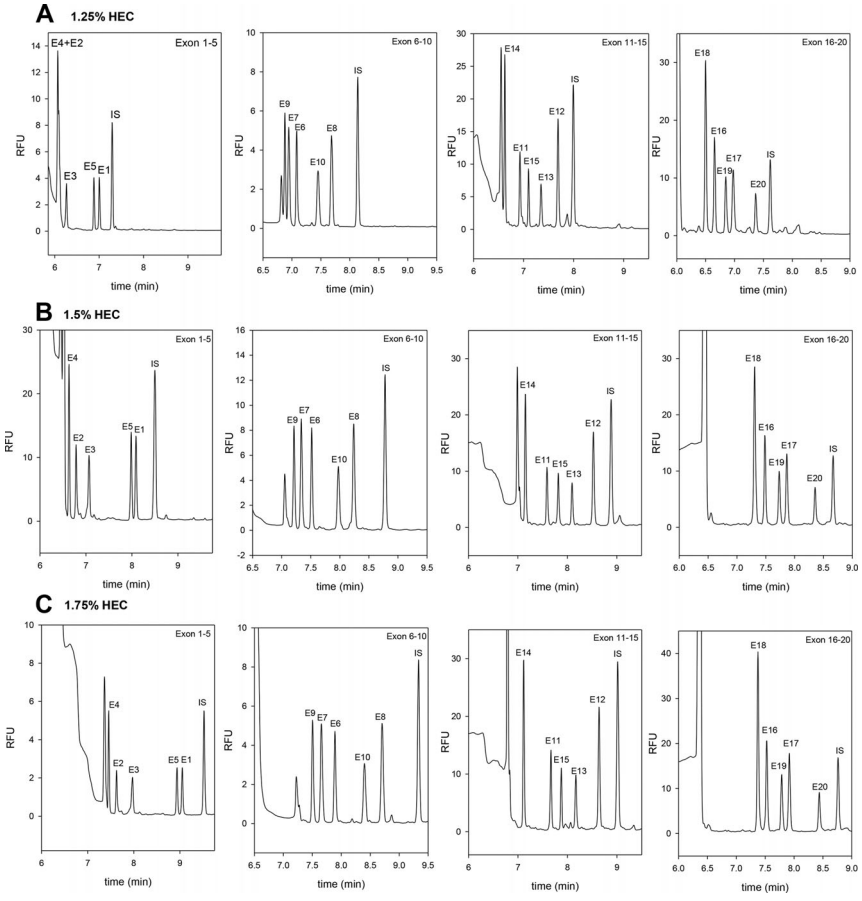

Figure 3. Effects of different concentrations of HEC polymer solutions on analysis of normal human sample. (A) 1.25% HEC, (B) 1.5% HEC, and (C) 1.75% HEC in 1× TBE buffer. Other conditions were as shown in Fig. 2. Peaks identification: IS: KRIT-1, E1–E20 represent exons 1–20, respectively.

only one universal FAM-labeled primer (uni-FAM) was required for fluorescently labeling all 20 exons and one internal standard (IS) genetic fragment. As shown in Fig. 1, genomic DNA was specifically amplified by the designed forward primers, which have a section of universal nonhuman sequence, and reverse primers. All of the intermediate amplicons possessed the universal sections by first eight cycles of PCR. Subsequently, the uni-FAM primer hybridized the complementary sequences of nonhuman genes and amplified them in the second 25 cycles of UMPCR. Finally, all DNA fragments were labeled with fluorophore. This method was found to be very helpful for detection of multiple genes.

3.2 Optimization of CE method

In order to obtain fast and efficient resolution, several parameters of the short-end CE were evaluated. Among them, types and concentration of polymer solutions, and separation voltage were found to be crucial.

Polymer solutions form different molecular sieves based on their native features. We tested 1.5% HEC, 1.5% HPMC, and 1.5% PEO, and showed the results in Fig. 2. When using 1.5% PEO, the viscosity of this solution is too high to inject the samples. Additionally, the peak of exon 4 coeluted with the peak of uni-FAM. The peaks of exons 1 and 5 in 1.5% HPMC could not be separated. HEC was the best polymer. Figure 3 showed the effects of different concentrations of HEC. Better resolution was observed when the concentration of HEC was higher than 1.5%. However, 1.75% HEC resulted in clogging due to high viscosity. Although the higher concentration of polymer solution gives better resolution, the higher viscosity is a difficult issue to perform operations. Finally, 1.5% HEC was selected.

A higher voltage can be used in CE than in slab gel electrophoresis, and results in better performance, speed, and high efficiency. The voltages of 100, 150, and 200 V/cm were investigated. As shown in Fig. 4, 150 V/cm (6 kV) was the optimal condition. The optimal condition is 1.5% HEC at 6 kV. Under the optimal conditions, the genetic fragments of exons 1–20 of DMD gene were well separated. However, there is an unidentified peak observed in the front of the target peaks. From the electropherogram of the blank (without adding genome DNA in UMPCR), the unidentified peak existed. The data were shown in Fig. 5. Therefore, this unidentified peak was supposed as a product formed by the annealing of the residual uni-FAM primer with other residual specific primers. Comparing with long-end injection (30 cm from the outlet of the capillary), short-end CE could save more than 20 min (data not shown).

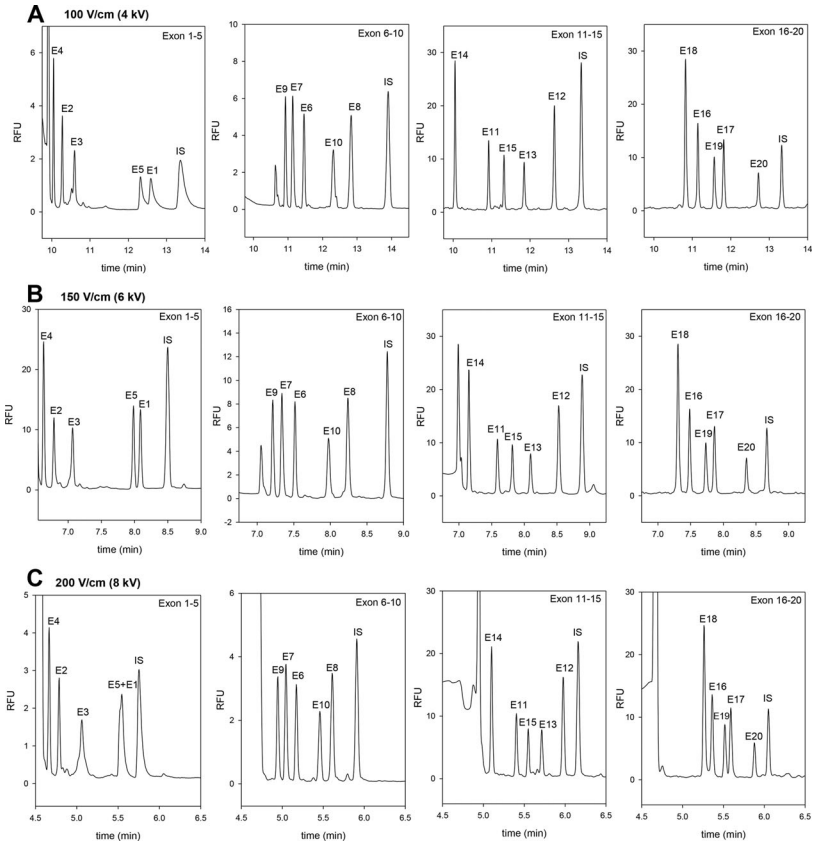

Figure 4. Effects of different separation voltages (A) 100 V/cm, (B) 150 V/cm, and (C) 200 V/cm. Other conditions were as shown in Fig. 2. Peaks identification: IS: KRIT-1, E1–E20 represent exons 1–20, respectively.

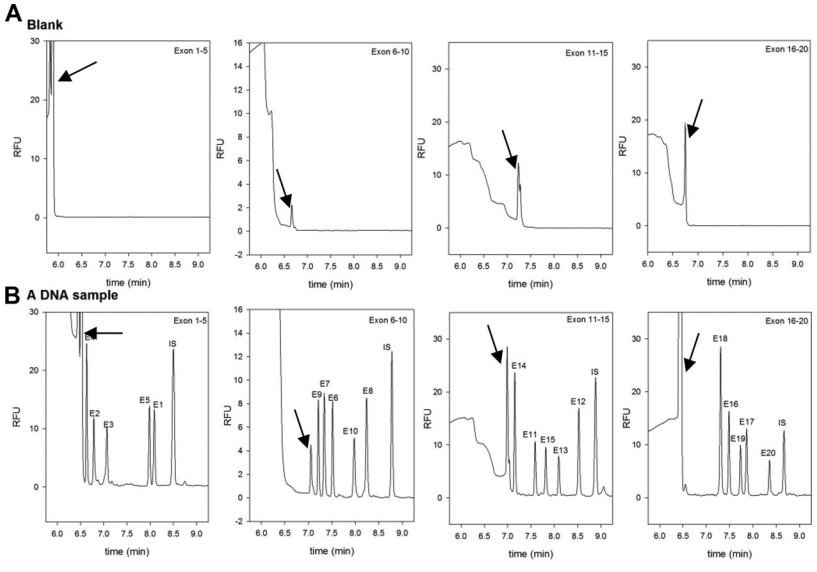

Figure 5. The electropherograms of the UMPCR products of (A) blank (without adding genome DNA in UMPCR) and (B) a DNA sample. The arrow indicated an unidentified peak resulting from the annealing of the uni-FAM primer with the specific primer. The optimal short-end CE conditions were as follows: sample injection, 10 kV for 20 s; separation voltage, 6 kV; separation matrix, 1.5% HEC in 1× TBE; capillary temperature, 25°C.

3.3 Applications

Seven genomic DNA samples were used to confirm the UMPCR method with short-end CE detection. Genomic samples were obtained from one healthy male volunteer (a normal genotypic person) and six male DMD patients. The six genomic DNA samples of the DMD patients have been analyzed by the MLPA method [4]. The electropherograms of these six DMD patients were shown in Fig. 6. The results of this UMPCR with short-end CE method were identical to MLPA, and indicated the good accuracy of this method.

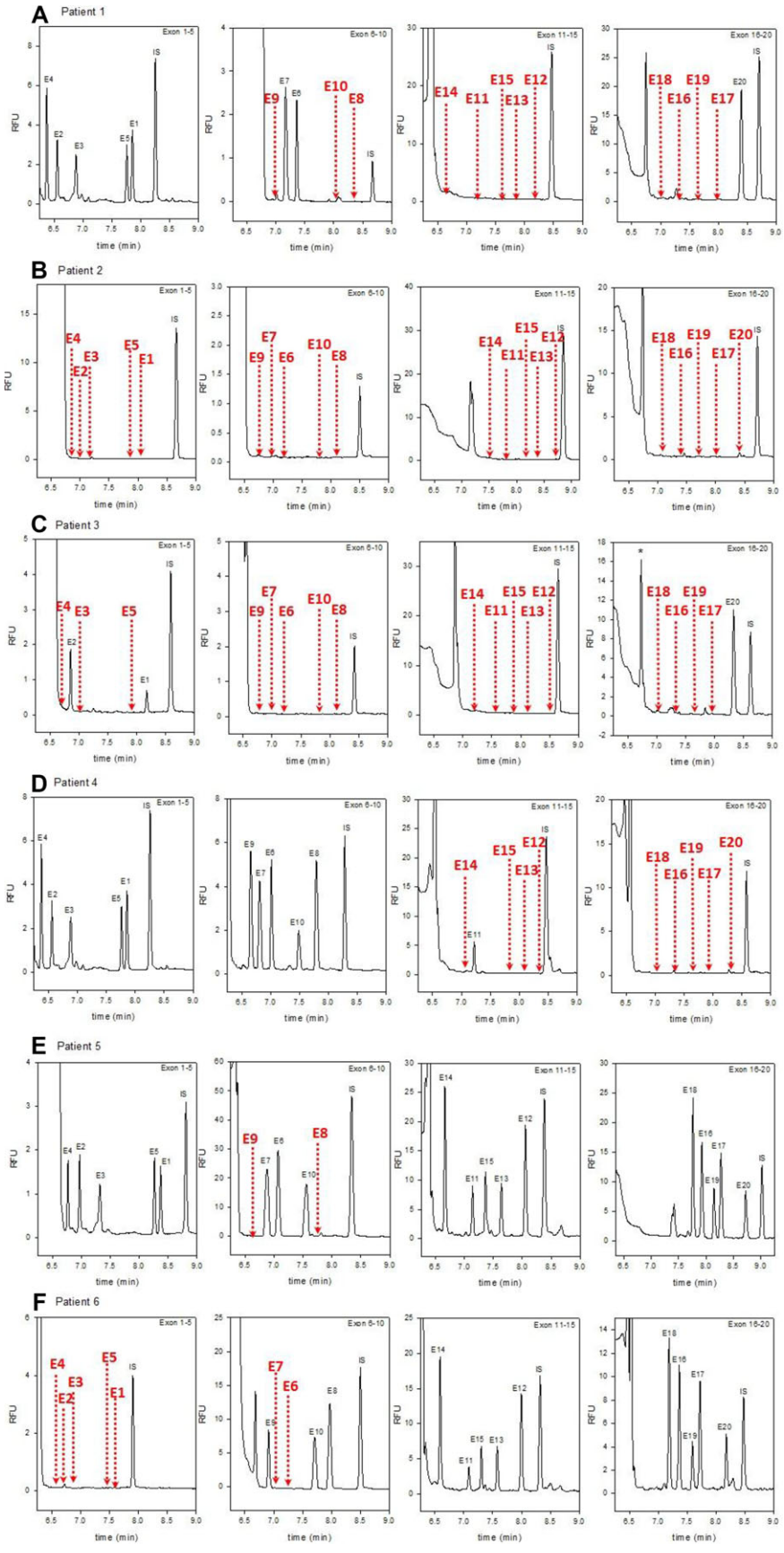

Figure 6. Electropherograms of six DMD patients analyzed by UMPCR and short-end CE method. The black-labeled numbers are expressed as the own exons of the DMD patient and the red-labeled numbers indicate the deletion exons of this patient. Peaks identification: IS: KRIT-1, E1–E20 represent exons 1–20, respectively. The data show that the DMD patient had the deletion of DMD gene on (A) exons 8–19 in patient 1, (B) exons 1–20 in patient 2, (C) exons 3–19 in patient 3, (D) exons 12–20 in patient 4, (E) exons 8–9 in patient 5, and (F) exons 1–7 in patient 6.

4 Concluding remarks

This study established an approach to analyze the DMD gene from exons 1–20 based on the UMPCR and short-end CE method. The cost of primer synthesis can be reduced greatly by using UMPCR and the separation time can be shortened by using the short-end CE method. The developed method was verified through six genomic DNA samples of DMD patients and the data of this strategy showed the good corresponding with that of the MLPA method, the most used technique for clinical detection of DMD. This simple, convenient, and fast method is feasible for using as a tool for diagnosis of DMD in a clinical assay.

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The authors have declared no conflict of interest.

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