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Universal fluorescent tri-probe ligation equipped with capillary electrophoresis for targeting *SMN1* and *SMN2* genes in diagnosis of spinal muscular atrophy

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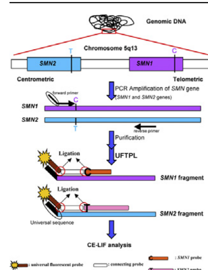
HIGHLIGHTS

- UFTPL-CE was used for detection of *SMN1* and *SMN2* genes in SMA.
- UFTPL was performed by adding three probes to differentiate SNP of *SMN*.
- Of the 48 samples, the data of gene dosages were corresponding to DHPLC methods.

GRAPHICAL ABSTRACT



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ABSTRACT

This is the first ligase chain reaction used for diagnosis of spinal muscular atrophy (SMA). Universal fluorescent tri-probe ligation (UFTPL), a novel strategy used for distinguishing the multi-nucleotide alternations at single base, is developed to quantitatively analyze the *SMN1/SMN2* genes in diagnosis of SMA. Ligase chain reaction was performed by adding three probes including universal fluorescent probe, connecting probe and recognizing probe to differentiate single nucleotide polymorphisms in UFTPL. Our approach was based on the two UFTPL products of *survival motor neuron 1* (*SMN1*) and *SMN2* genes (the difference of 9 mer) and analyzed by capillary electrophoresis (CE). We successfully determined various gene dosages of *SMN1* and *SMN2* genes in homologous or heterologous subjects. By using the UFTPL-CE method, the *SMN1* and *SMN2* genes were fully resolved with the resolution of 2.16 ± 0.37 ($n = 3$). The r values of *SMN1* and *SMN2* regression curves over a range of 1–4 copies were above 0.9944. Of the 48 DNA samples, the data of gene dosages were corresponding to that analyzed by conformation sensitive CE and denatured high-performance liquid chromatography (DHPLC). This technique was found to be a good methodology for quantification or determination of the relative genes having multi-nucleotide variants at single base.

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1. Introduction

Single-nucleotide polymorphisms (SNPs) are the most frequent genetic variant in humans, and often occur biallelically at a rate of approximately once every 100–300 bases [1,2]. A lot of individual SNPs located in coding regions have been already known to affect

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gene biological functions and cause several genetic diseases [3–6]. Therefore, highly selective and sensitive detection of SNPs is a requisite for heredity-related risk assessment, disease diagnostics and drug development. Several techniques have been developed for detecting SNPs, typically including polymerase chain reaction (PCR) [7,8], primer extension [9,10], strand displacement amplification (SDA) [11,12], rolling circle amplification (RCA) [13–15] and ligase chain reaction (LCR) [16–18]. LCR offers a sensitive and specific alternative platform for detection of SNPs [16–21]. In this study, a novel method of universal fluorescent tri-probe ligations (UFTPL) is developed for analyzing the multi-nucleotide difference at single base. The mechanism of this method is similar to LCR. LCR is a method of DNA amplification requiring the nucleic acid as the probes for each of the two DNA strands and then two partial probes are ligated to form the actual one. The LCR needs two enzymes of DNA polymerase and DNA ligase, and is more specific compared to PCR [22]. Our UFTPL method utilized three probes to provide higher specificity than LCR, and was carried out by only using the DNA ligase without the DNA polymerase. It is superior to LCR due to specific recognition of SNPs and more sensitive fluorescent labeling. This method was applied for quantitative determination of survival motor neuron genes (*SMN*) in diagnosis of spinal muscular atrophy (SMA).

SMA is an autosomal recessive disease characterized by degeneration of motor neurons which results in progressive muscular atrophy and weakness [23,24]. The severe disease has high incidence of 1 in 6000–10,000 live births and the carrier rate is 1:35 [25,26]. Two SMA-determining genes, a telomeric *SMN1* (MIM# 600354) and a centromeric *SMN2* gene (MIM# 601627), were located at chromosome 5q13. Most of SMA patients (95%) contract the disease by either deletion or conversion of at least exons 7 and 8 of the telomeric *SMN1* gene [27,28]. The sequences of *SMN1* and *SMN2* genes are extremely similar with only five nucleotide differences, including cDNAs-substitution of single nucleotides in exons 7 (c.840 C > T) and 8 (G > A). The mutation in exon 7 renders the *SMN2* gene incapable of compensating for the *SMN1* gene, but allows the *SMN1* gene to be distinguished from the *SMN2* gene. Thus, detection of the absence of *SMN1* exon 7 is a powerful tool for pre- and post-natal diagnosis of SMA.

Until now, many strategies have been established for determination of *SMN1*/*SMN2* genes, such as quantitative real-time PCR [24,29], DHPLC [30], MALDI-TOF mass spectrometry [31] or capillary electrophoresis (CE) [32,33]. These methods are simple, but misdiagnosis may occur when an extra nucleotide variant happened on the genetic fragments. In addition to utilization of these strategies, some other helpful techniques have also been employed for resolution of *SMN1*/*SMN2* genes. Single-base extension (SBE) or primer extension, known as minisequencing, is widely used for SNP typing [34,35]. The SBE primer is only extended one base because the ddCTP or ddTTP acts as a terminator in the extension reaction [34]. Primer extension was performed in the presence of a mixture of dATP, dGTP, dTTP and ddCTP, resulting in 4 base pairs of different lengths, between *SMN1* and *SMN2* [35]. However, SBE or primer extension may cause the difficult recognition between *SMN1* and *SMN2*. On the other hand, UFTPL could easily identify *SMN1* and *SMN2* by designing probes with different base numbers. In this study, a new methodology of UFTPL coupled with CE was developed to determine *SMN1*/*SMN2* gene dosage.

2. Experimental

2.1. Materials

Hydroxyethyl cellulose (HEC, 1% in H_2O ~145 mPa s) was purchased from Fluka (Fluka Chemie Gmbh, Switzerland). Hydroxypropyl cellulose (HPC, M.W. ca. 80,000) was obtained from Sigma–Aldrich (Sigma, St. Louis, MO, USA). Methanol and urea were purchased from Merck (Merck, Darmstadt, Germany). 5× TBE buffer was purchased from Protech Technology Enterprise Co., Ltd. and diluted with dd-water before use. The dd-water was obtained from a Milli-Q water system (Millipore, Bedford, MA, USA).

2.2. Genomic DNA samples

The ethics approval for this study was obtained from the Institutional Review Board at Kaohsiung Medical University Hospital where participants were recruited and experiments on humans were conducted. Written informed consents were obtained from all participants. Total of 48 DNA samples were analyzed by this UFTPL method, which included 10 SMA patient samples, 12 parent samples of these 10 patients, and 26 normal controls. The phenotypes were differentiated by the doctors in the hospital. Genomic DNA was collected from peripheral whole blood through using GFX™ Genomic Blood DNA Purification kit. The *SMN1*/*SMN2* ratios of all DNA samples were quantitatively analyzed by the UFTPL method.

2.3. Amplification of SMN fragments

In this research, the *SMN* gene fragments including *SMN1* and *SMN2* and a *KRIT1* gene fragment serving as internal standard (IS) for determining the relative gene dosage of *SMN1*/*SMN2* were simultaneously amplified by PCR. The primers used in PCR were as shown in Table 1. A fluorescent dye was used to label the primer of *KRIT1* gene in order to make it detectable by a fluorescent detector. The total volume of 50 μL for PCR contained 200 ng of genomic DNA, 0.16 μM of each primer for the *KRIT1* gene and 0.2 μM of each primer for the *SMN* gene, 200 μM dNTPs, 0.2 μL of e2TAK DNA polymerase (TaKaRa Biotechnology Co., Ltd.), and 10 μL of 5× e2TAK PCR buffer as provided by the manufacturer. The PCR amplification was performed in a Px2 thermo cycler (Thermo Electron Co.) with an initial denatured step at 95 °C for 10 min, followed by 25 cycles consisting of denaturation at 95 °C for 45 s, annealing at 53 °C for 45 s, extension at 72 °C for 1 min and a final extension step at 72 °C for 10 min. After completion of PCR, the products were cleaned up by a DNA Clean/Extraction kit (Gene-Mark, Hopegen Biotechnology Development Enterprise). The purified DNA was stored at −20 °C prior to the UFTPL reaction.

2.4. Universal fluorescent tri-probe ligation (UFTPL)

In the UFTPL reaction, a total volume of 25 μL contained 5 μL of the purified DNA product, 2.5 μL of 10× *Taq* DNA ligase reaction buffer (200 mM Tris–HCl, 250 mM potassium acetate, 100 mM magnesium acetate, 10 mM NAD, 100 mM dithiothreitol and 1% Triton X-100 at pH 7.6), 20 nM universal fluorescent probe (UF

Table 1
Primers used in amplification of *SMN* gene and UFTPL reaction.

Primer name	Oligonucleotide sequence (5′#5–4#)
KRIT1-F	FAM-TTCGAATGGCTACTTCTACCTG
KRIT1-R	AAAACGTCTTTTAAATCAGAGC
SMN-F	ATAAGTGACGTACTAGCAACGTTCCTTTATTTTCCTTACAGGGTTT
SMN-R	TTCCACAAACCATAAAGTTTT
UF probe	FAM-GTGACGTACTAGCAACG
Connecting probe	PO_4-TTCCTTTATTTTCCTTACAGGGTTT
SMN1 probe	PO_4-CAGACAAAATCAAAAAGAAG
SMN2 probe	PO_4-TAGACAAAATCAAAAAGAAGGAAGGTGCT

FAM: 6-Carboxy-fluorescein was the fluorescent dye for LIF detection.
PO_4: Phosporylation on the 5′-end.

probe), 20 nM connecting probe, 20 nM *SMN1* probe, 2 nM *SMN2* probe and 40 U of *Taq* DNA ligase (NEW ENGLAND BioLabs®, 240 County Road, Ipswich). The ligation was carried out at 95 °C for 3 min, 65 °C for 2 h and 95 °C for 10 min. The products were directly analyzed in CE system after 10-fold dilution with dd-water.

2.5. CE system

UFTPL products were analyzed in a coated DB-17 capillary (Agilent Technologies Co.) of 100 μm internal diameter and 30 cm effective length. Before analysis, capillary was pre-washed with MeOH for 10 min and dd-water for 20 min. Then the separation matrix consisting of a mixture of 1.5% HEC, 2.0% HPC in 2.0× TBE buffer was injected at 40 psi for 50 min. Between runs, the separation matrix was re-rinsed at 40 psi for 10 min. The CE instrument was the Beckman P/ACE MDQ system (Fullerton, CA, USA) equipped with a laser induced fluorescence detector which utilized a laser energy (488 nm) for exciting the electrons of materials to obtain the fluorescence (excitation wavelength: 488 nm; emission wavelength: 520 nm). Sample injection was carried out at −10 kV for 30 s and then the separation was accomplished at 8 kV in the reverse polarity mode. The temperature was normally set at 15 °C.

2.6. Determination of SMN1/SMN2 copy numbers

The *KRIT1* gene fragment (IS, 327 bp) was used to obtain the number of copies of *SMN1* and *SMN2* genes in exons 7 through comparing the peak height of *SMN1* and *SMN2* with that of IS genetic fragments. Standards of known copies were applied to establish the calibration curve which was further used for calculation of *SMN1*/*SMN2* ratios of the blind samples. Because the UFTPL of *SMN* gene is integral duplication, thus, the calibration curves were established by using of standards having one, two,

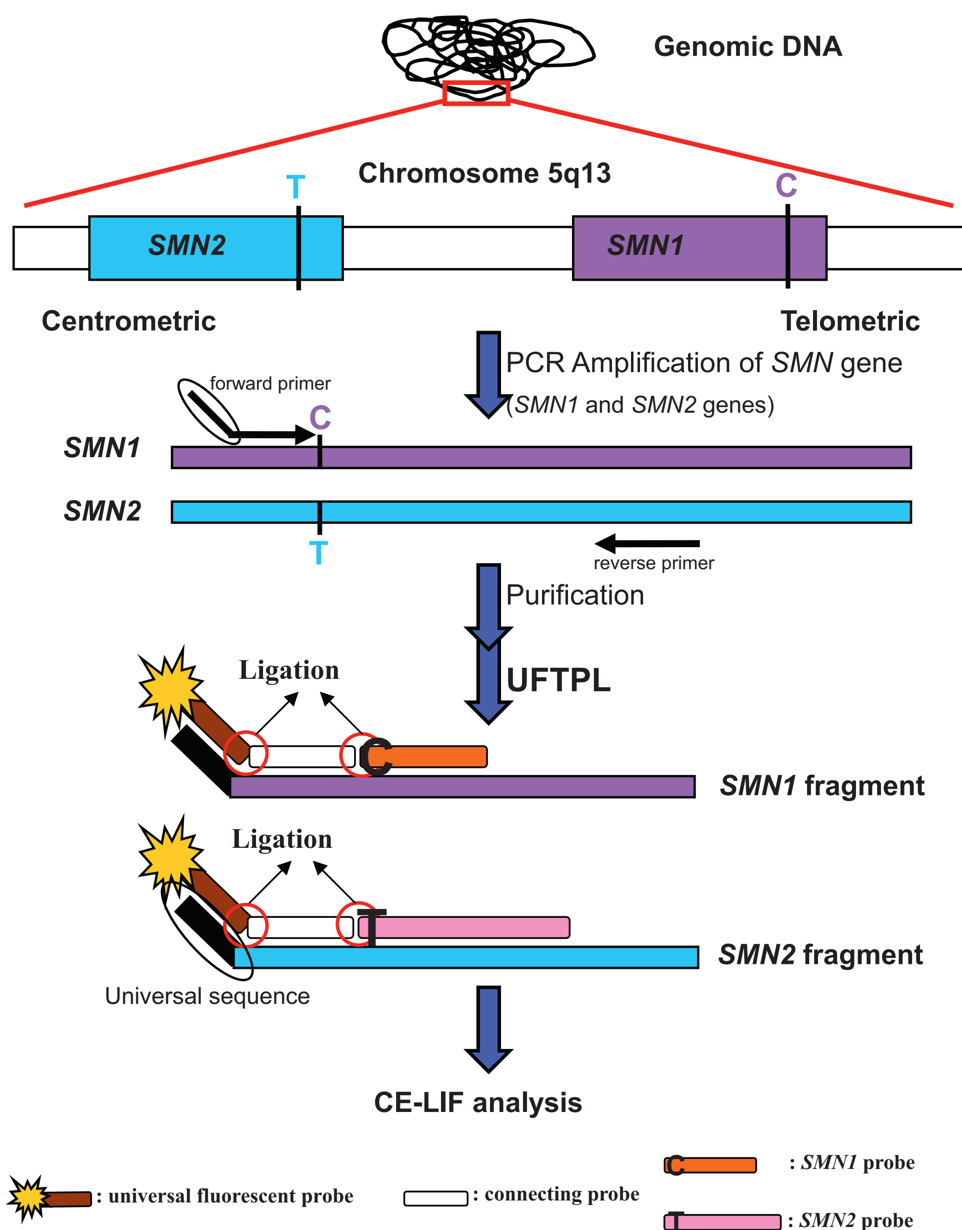


Fig. 1. The schema of UFTPL for analysis of *SMN1* and *SMN2* genes.

three and four copy of *SMN1* and *SMN2* genes, respectively. The detection limit of this method was one copy of *SMN1* or *SMN2* gene. Data were taken for all participants, including normal phenotype controls, carriers and SMA patients.

3. Results and discussion

3.1. UFTPL assay

This study used universal fluorescent probe (UF probe), connecting probe and recognizing probe for universally fluorescent labeling and rapidly determining *SMN1*/*SMN2*. The mechanism was as shown in Fig. 1. Initially, the *SMN* genes including *SMN1* and *SMN2* were amplified by a pair of primers where one contained a part of universal sequences. After PCR, all *SMN1*/*SMN2* fragments possessed the universal sequence and the products were purified by a clean-up kit for UFTPL reaction. In UFTPL, three probes were used to establish the individual fluorescent fragments with different lengths. By designing unique sequences, the universal fluorescent probe and *SMN1*/*SMN2* probes attached to the universal sequence and the specific sequence of *SMN1*/*SMN2*, respectively. The *SMN1* and *SMN2* probes having different lengths (difference of 9 mer) were used to individually recognize the *SMN1* and *SMN2* genes. Then, the three probes were linked by the ligation enzyme. The procedures of hybridization and ligation increase the specificity for detection of *SMN1* and *SMN2* and FAM labeling enhanced the sensitivity of DNA detection. Finally, fluorescently labeled single-strand *SMN1* and *SMN2* fragments with different lengths could be easily separated and detected by CE-LIF.

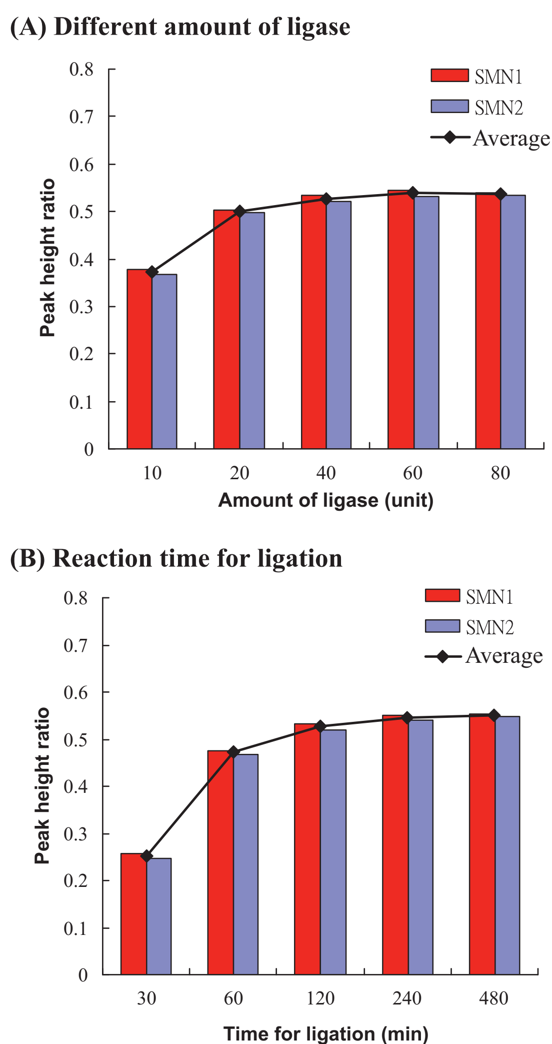


Fig. 2. Effects of (A) the amount of ligase enzyme and (B) reaction time for ligation on the *SMN1*/*SMN2* peak height ratios. (Peak height ratios: the peak height of *SMN1* or *SMN2*; the peak height of IS).

3.2. Optimization of UFTPL reaction

The ligation of oligonucleotides plays an important role in the UFTPL reaction. In this study, a case of *SMN1*/*SMN2* equal to 2/2 was used for investigating the amount of ligase and time for ligation, respectively (Fig. 2). Fig. 2A shows the peak height ratios of *SMN1* and *SMN2* fragments compared to IS when adding 10, 20, 40, 60 and 80 units of ligase in 25 μL reaction volume. Considering the reaction efficiency, 40-unit of ligase has reached the maximum reaction. Therefore, 40-unit of ligase was chosen as the optimal condition. The data of reaction time for ligation was as shown in Fig. 2B. Finally, the ligation reaction was accomplished within 120 min. The peak of *SMN2* was higher than *SMN1*, when the same amounts of *SMN1* and *SMN2* probes were added for ligation. In order to unify the peak height of *SMN1* and *SMN2* on the cases possessing equivalent gene ratio (1:1 or 2:2), the amount of *SMN2* probe was reduced to obtain the same peak height of *SMN1* and *SMN2*. At last, the amount of *SMN2* was lower than *SMN1* by 10-fold.

3.3. Evaluation of CE system

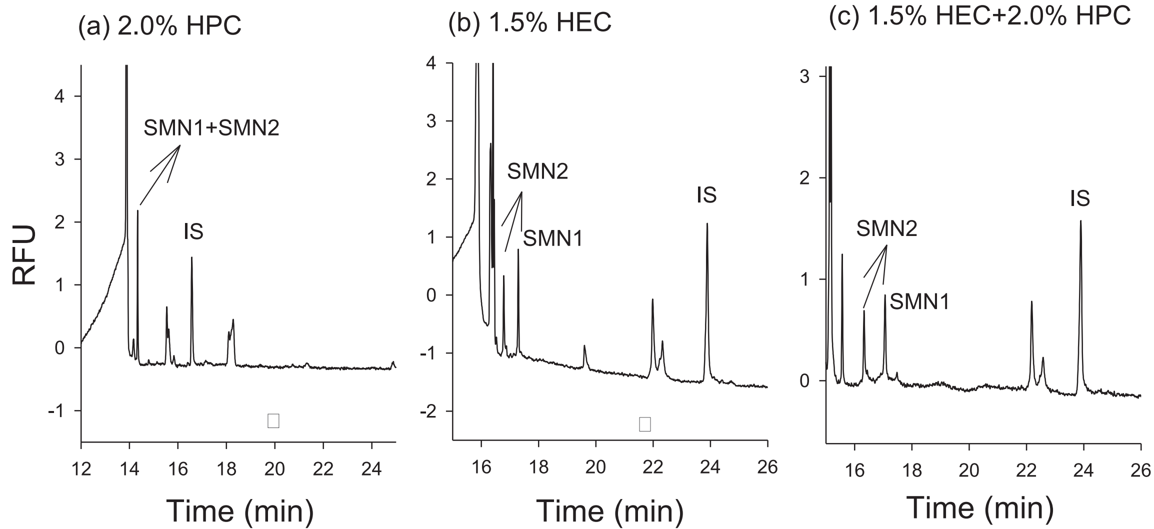
In order to obtain efficient resolution of *SMN1* and *SMN2* single-strand fragments, different compositions of the polymer solution and various capillary temperatures were investigated. Fig. 3A shows the effect of the polymers on the resolution of *SMN1*/*SMN2* genes. Single polymer solution of 2% HPC could not be used for analysis of *SMN1*/*SMN2*, when compared to the mixture of 2% HPC and 1.5% HEC. The combination of polymers formed a new separation matrix taking advantage of the best qualities of each polymer and generated a quasi-interpenetrating network which could provide more efficacy than single polymer solutions [36]. Finally separation was performed by using the mixed polymer solution of 2% HPC and 1.5% HEC. As to the capillary temperature, low temperature provided a better resolution of *SMN1*/*SMN2* than others (Fig. 3B), due to the viscosity and the rigid structure of the polymers at low temperature. Therefore, 15 °C was used.

3.4. UFTPL with CE analysis

The UFTPL is able to easily differentiate between *SMN1* and *SMN2* genes. Therefore, when compared to other methods used to produce the variance of *SMN1* and *SMN2* amplicons, such as SBE or primer extension [34,35], UFTPL is able to more easily recognize and resolve *SMN1*/*SMN2* genes by CE analysis. Under the optimal CE conditions, the *SMN1* and *SMN2* peaks were completely baseline separated and resolution was 2.16 ± 0.37 (n = 3), calculated through Eq. (1).

$$\mathrm{Rs} = \frac{2(t_B - t_A)}{W_A + W_B} \quad (1)$$

The migration time of *SMN1* peak was about 17.23 ± 0.25 min and that of *SMN2* was about 16.53 ± 0.17 min. Although the UFTPL product of *SMN2* was longer than *SMN1* by 9 mer, the *SMN2* fragment migrated faster than *SMN1*. The single-strand structures of UFTPL products of *SMN1* and *SMN2* twist irregularly and thus the migration order could not be judged by the length. Fig. 4 displays the electropherograms and sequencing data of individuals



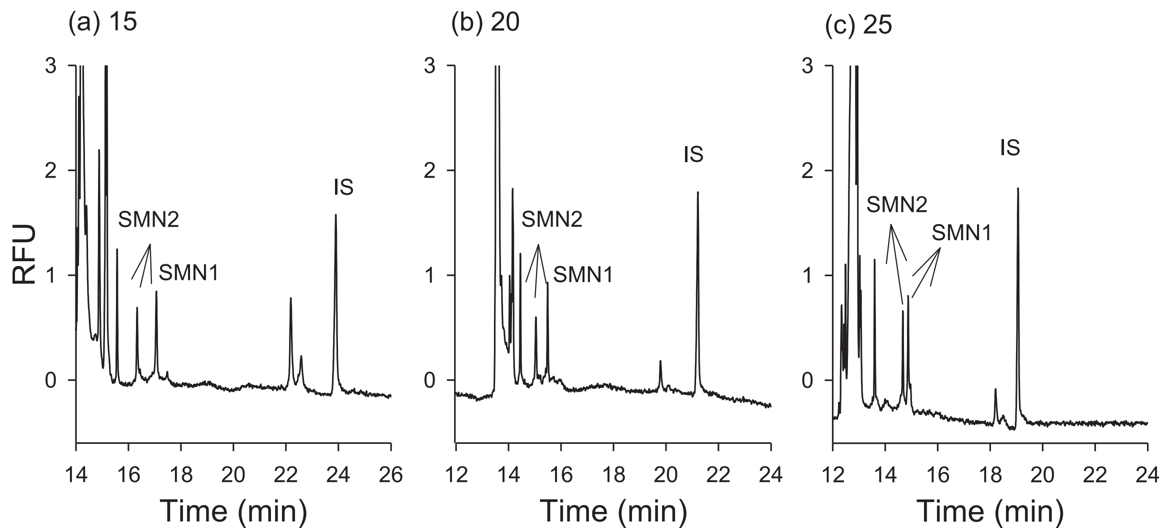


Fig. 3. Electropherograms of (A) different polymers of (a) 2.0% HPC in 2× TBE; (b) 1.5% HEC in 2× TBE and (c) 1.5% HEC + 2.0% HPC in 2× TBE. (B) Various capillary temperatures of (a) 15 °C; (b) 20 °C and (c) 25 °C. The analyte was the UFTPL product obtained from a subject possessing *SMN1*/*SMN2* ratio of 2:2. Other CE conditions were as follows: sample injection, −10 kV for 30 s; separation voltage, −8 kV.

possessing only *SMN1* gene, only *SMN2* gene and both genes. The data indicated that the front peak was referred to *SMN2* gene and the back peak was referred to *SMN1* gene.

3.5. Various ratios of SMN1/SMN2

In this research, various copy numbers of *SMN1* and *SMN2* genes were analyzed by the UFTPL-CE method. Fig. 5A displays the electropherograms of subjects having different ratios of *SMN1*/*SMN2*. The UFTPL-CE technique was successfully applied to resolve and quantify the *SMN1* and *SMN2* genes. After calibrating the *SMN1* and *SMN2* copy numbers versus the peak height ratio by using the standard samples, the regression curves were established. Fig. 5B shows a linear relationship between the observed peak height ratios and the known *SMN1* and *SMN2* copy numbers. Regression equations of *SMN1* and *SMN2* were $y = 0.3119x - 0.0372$ ($r = 0.9944$) and $y = 0.3625x - 0.1422$ ($r = 0.9949$), respectively ($n = 3$). The formulas were further applied for calculating the *SMN1*/*SMN2* gene ratios of the analyzed population.

3.6. Applications

In this research, a total of 48 DNA samples were analyzed by the UFTPL-CE method. The various *SMN1*/*SMN2* ratios among the 48

participants were as shown in Table 2. In the group of SMA patients, 2 had the *SMN1*/*SMN2* ratio of 0:4, 5 had 0:3 and 3 had 0:2. Among the carriers who were the parents of these SMA patients, the number of subjects possessing *SMN1*/*SMN2* ratio of 1:1 was 2, of 1:2 was 5 and of 1:3 was 5. Of the 26 normal controls, *SMN1*/*SMN2* ratios of 2:1, 2:2, 3:0 and 4:0 included 8, 16, 1 and 1 subjects, respectively. The other two methods of conformation sensitive capillary electrophoresis (CSCE) [32] and DHPLC [37] were also used for verifying, and the data were corresponding to those of this UFTPL-CE method. This method was demonstrated successfully to be a viable tool for quantification of *SMN1*/*SMN2* in diagnosis of SMA.

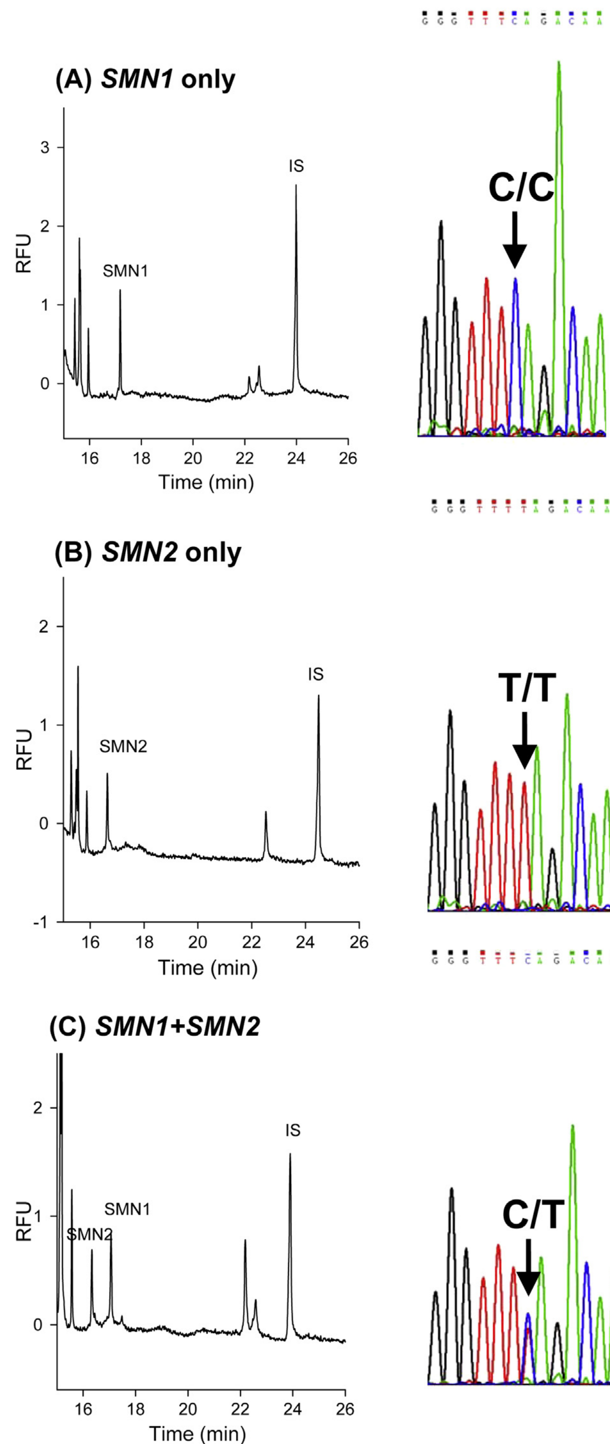
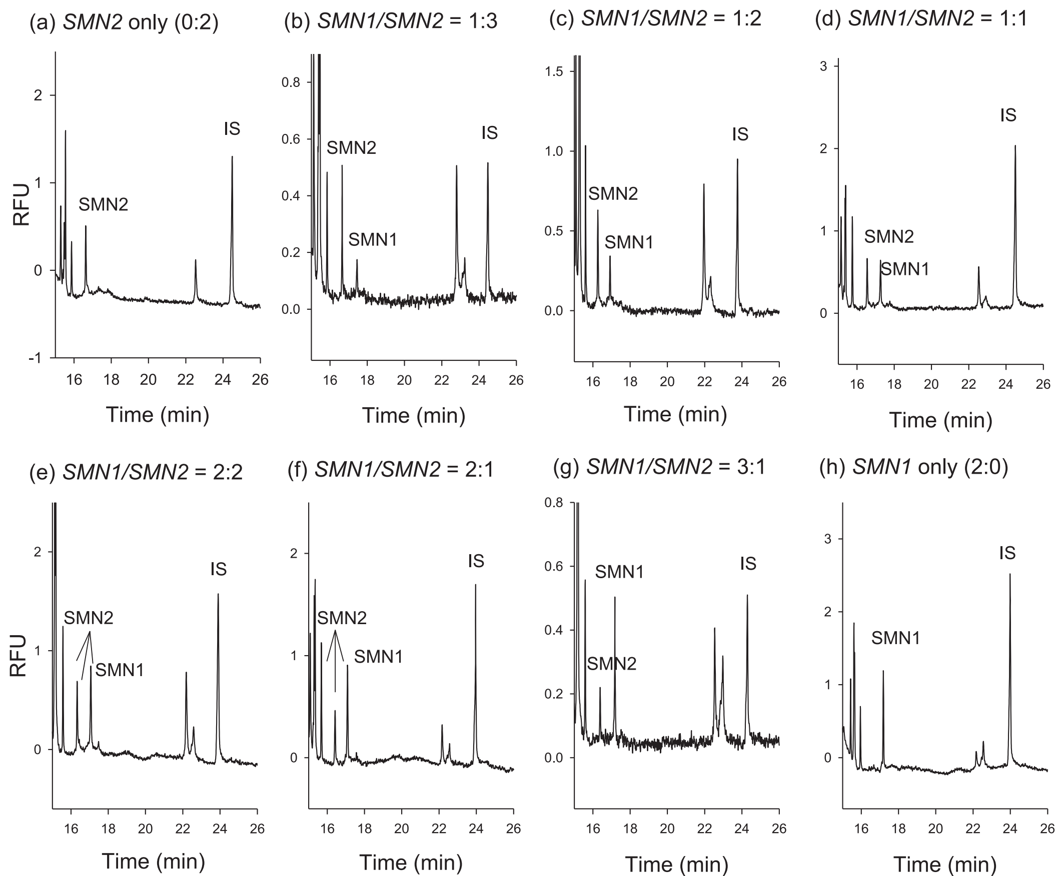


Fig. 4. CE electropherograms and sequencing analysis of UFTPL products of (A) an individual with only *SMN1* gene; (B) an individual with only *SMN2* gene and (C) an individual with a gene ratio of *SMN1*/*SMN2* equaled to 2:2.



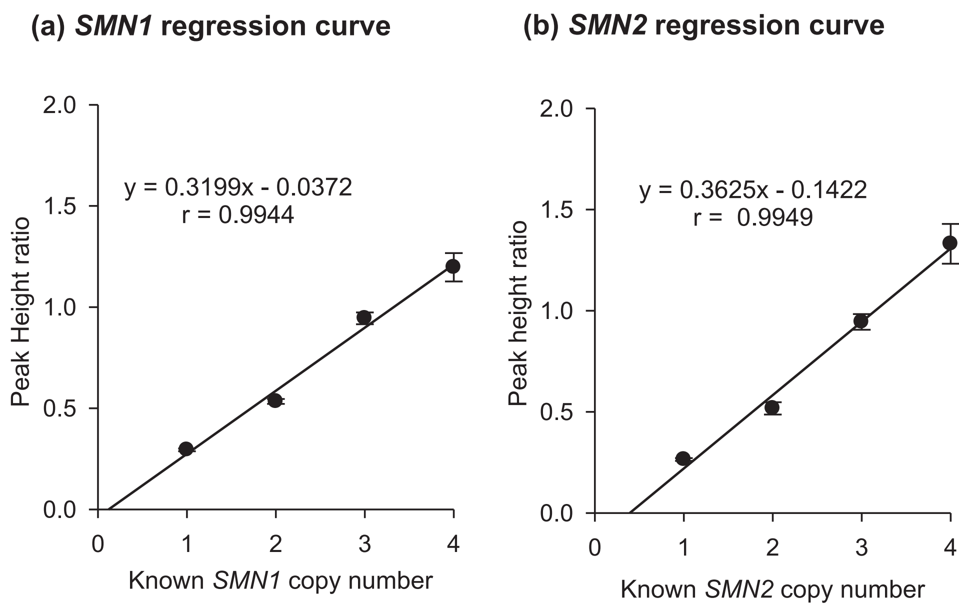


Fig. 5. (A) CE electropherograms for analysis of UFTPL products obtained from different DNA samples with various *SMN1*/*SMN2* gene ratios. (B) The linear relationship between (a) *SMN1* and (b) *SMN2* peak height ratios (compared with IS) versus the known copy number of (a) *SMN1* and (b) *SMN2*.

Table 2
Data of different *SMN1*/*SMN2* ratios analyzed by the UFTPL-CE method among the 48 subjects.

Genotype (SMN1/ SMN2)	Copy number detected by UFTPL-CE	Number of subjects	Statue	CSCE method	DHPLC method
0:4	0.00 ± 0.00:3.88 ± 0.10	2	SMA	○	○
0:3	0.00 ± 0.00:3.15 ± 0.16	5	SMA	○	○
0:2	0.00 ± 0.00:2.15 ± 0.10	3	SMA	○	○
1:1	1.05 ± 0.04:1.11 ± 0.08	2	Carrier	○	○
1:2	1.04 ± 0.06:2.04 ± 0.07	5	Carrier	○	○
1:3	1.02 ± 0.10:2.96 ± 0.08	5	Carrier	○	○
2:1	1.98 ± 0.13:1.06 ± 0.09	8	Normal	○	○
2:2	2.02 ± 0.13:1.92 ± 0.08	16	Normal	○	○
3:0	2.93:0	1	Normal	○	○
2:0	2.07:0	1	Normal	○	○
Total		48			

4. Conclusions

A novel UFTPL-CE method was successfully established for determination of *SMN1* and *SMN2* genes. The important advantages of this method are better resolution by adding an extra probe for differentiation, and using universal fluorescent primer. This method is expected to be extended for quantification or separation of the relative genes having multi-nucleotide variants at single base, such as *K-ras*, *EGFR* or others.

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