

Determination of *SMN1*/*SMN2* Gene Dosage by a Quantitative Genotyping Platform Combining Capillary Electrophoresis and MALDI-TOF Mass Spectrometry

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Background: Spinal muscular atrophy (SMA) is a common inherited and fatal neuromuscular disease caused by deletions and/or mutations that lead to altered concentrations of proteins encoded by the survival motor neuron genes *SMN1* and *SMN2*. Because of the high incidence (at least 1 in 10 000 live births and a carrier frequency of 1 in 35 to 1 in 50) and severity of the disease, precise quantification of *SMN1* and *SMN2* gene copy numbers is essential for diagnosis and genetic counseling.

Methods: We developed a genotyping platform combining capillary electrophoresis and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) to quantify absolute gene dosage. The absolute gene dosage can be determined by a multiplexed competitive PCR protocol followed by capillary electrophoresis analysis. The relative *SMN1*/*SMN2* ratio can be analyzed by PinPoint assay followed by MALDI-TOF MS analysis.

Results: The complementary assays were evaluated in confirmed cases including 9 affected patients, 33 carriers, and 478 healthy individuals from the general population. We were able to determine all genotypes with different *SMN1*/*SMN2* gene copy number ratios, which

unambiguously diagnosed carrier status and the severity of SMA with 100% specificity.

Conclusions: This quantitative genotyping platform is suitable for detection of SMA. The described approach may serve as a general quantitative genotyping method for molecular diagnosis of other inheritable diseases.

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Spinal muscular atrophy (SMA),⁶ one of the most common autosomal recessive disorders, has an estimated incidence of at least 1 in 10 000 live births and a carrier frequency of 1 in 35 to 1 in 50 (1, 2). The disease is caused by a loss of α -motor neurons in the spinal cord anterior horn cells and results from mutations in the survival motor neuron (*SMN*) genes. Patients with SMA are classified into 3 groups based on the clinical severity of the disease: severe, intermediate, and mild (SMA type I, II, and III, respectively) (3). Type I SMA (Werdnig–Hoffman disease; MIM# 253300) is the most severe form, with clinical onset generally occurring before the age of 6 months and death in the first 2 years of life. Type II SMA (MIM# 253550) is of intermediate severity and causes some patients to be unable to walk. Type III SMA (Kugelberg–Welander disease; MIM# 253400) is a mild form of the disease with onset after the age of 18 months (4), and with patients gaining the ability to walk.

The SMA-determining gene group, *SMN*, is located in a complex region of chromosome 5q13 and includes 2 highly homologous genes, telomeric *SMN1* (MIM# 600354) and centromeric *SMN2* (MIM# 601627). The 2 closely related genes differ at only 5 bp within their

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⁶ Nonstandard abbreviations: SMA, spinal muscular atrophy; SMN, survival motor neuron; HDA, high-performance DNA analysis; LED, light-emitting diode; SNP, single-nucleotide polymorphism; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; dNTP, deoxynucleoside triphosphate; MBS, multiblock system; and TEAA, triethylammonium acetate.

3'-terminal regions. Among the differences in exons 7 and 8, substitution of a single nucleotide (c.840C>T) decreases the activity of an exonic splicing enhancer. As a consequence, lower amounts of full-length transcript are generated from *SMN2* than from *SMN1*, leading to a deficiency of normal, stable SMN protein (5, 6).

Deletions of the functional *SMN1* appear to be directly involved in SMA; approximately 94% of clinically typical SMA-affected patients lack both copies of *SMN1* exon 7 (7). Moreover, small deletions or point mutation that cause gene conversion from *SMN1* to *SMN2* have been found in patients in whom *SMN1* was present (8). The clinical phenotypes of SMA depend on the mutation mechanism. Although absence of the *SMN2* allele cannot cause SMA, the *SMN2* copy number correlates inversely with disease severity and length of survival (9, 10). Potential therapies for SMA include approaches to increase expression of full-length *SMN2* transcripts (11); however, *SMN2* cannot compensate for *SMN1* deletion/conversion. Hence, identification of *SMN1* deletions and determination of *SMN1* and *SMN2* copy numbers are of clinical importance in genetic counseling and are of therapeutic significance.

The high-performance DNA analysis (HDA) system, a 12-channel capillary electrophoresis instrument equipped with a disposable cartridge for rapid DNA separation and detection, was developed recently (12). The HDA system uses superbright light-emitting diodes (LEDs) based on InGaN material technology, which allows transmission of 524 nm excitation light from the LEDs to gel-filled multicapillaries. In contrast to all previous capillary electrophoresis instruments, the microoptical collector in the HDA system can improve detection sensitivity. Therefore, HDA provides a non-fluorescence-based approach with sensitive, inexpensive, and high-throughput feature.

For single-nucleotide polymorphism (SNP) analysis, primer extension (minisequencing) is a direct method for multiplexed high-throughput genotyping using allele-specific DNA products. Minisequencing is the most widely used strategy for SNP genotyping because it provides high specificity and sensitivity with use of various detection techniques (13, 14). Compared with other SNP analysis methods, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) offers direct and accurate differentiation of the allele-specific extension products (15). Furthermore, an additional advantage of high-throughput MALDI-TOF MS analysis is that simultaneous multiplexed genotyping can be achieved in a single experiment (16). Therefore, MALDI-TOF MS in combination with a minisequencing strategy has become one of the most promising tools for mutation analysis to allow study of polymorphisms in disease-causing genes (17, 18).

The capability of the MALDI-TOF MS-based genotyping approach has been reported for quantitative SNP analysis using standard mixtures of pooled PCR products at several test loci (19). In this study, single-base primer

extension by the addition of a dideoxynucleotide (PinPoint assay) yields allele-specific products that differ in relative molecular mass (M_r) by 9 to 40 units; this small mass difference is difficult to differentiate by routine low-resolution MALDI MS in the presence of trace cation (NH_4^+ , Na^+ , and K^+) adducts. Therefore, in that study, a very short extension strategy using alternating deoxynucleoside triphosphates (dNTPs) and dideoxynucleoside triphosphates was adopted so that the products differed in mass by M_r 300 to 400 to resolve the 50/50 heterozygote for direct quantification from mass spectra. In addition, a calibration curve had to be constructed to correct the mass spectra response. We previously reported a sequential desalting method (20) to decrease cation adducts and consequently increase the required resolution for the multiplexed PinPoint assay (21) in mass analysis. Among all minisequencing methods, the PinPoint assay produces the smallest extension products, which are least susceptible to variations in extension efficiency (22). The PinPoint assay is therefore presumed to provide accurate relative quantification of gene dosage based on the smallest difference in primer extension products.

Here we report a quantitative genotyping platform that combines capillary electrophoresis for determining absolute gene dosage and the PinPoint assay and MALDI-TOF MS for quantitative SNP analysis. With respect to SMA diagnostics, we evaluate the method using the critical C-to-T substitution at position +6 of exon 7 (c.840C>T) in SMA for differentiation between *SMN1* and *SMN2*. The copy numbers and relative ratio of the *SMN1* and *SMN2* genes obtained with this platform can be determined to diagnose the status of SMA carriers in the general population. We confirmed the reliability of the platform in a study of 9 SMA-affected patients, 33 SMA carriers, and 478 healthy individuals. Our results demonstrate the feasibility of rapid and accurate quantitative genotyping for high-throughput, unambiguous diagnosis of SMA.

Materials and Methods

PATIENT SAMPLES

A total of 520 DNA samples were analyzed in this study, including samples from patients diagnosed with SMA, from carriers (i.e., families of SMA patients), and from apparently healthy individuals from the general population. The donors of all samples gave informed consent. Genomic DNA was collected from peripheral whole blood with a Puregene DNA Isolation Kit (Gentra Systems, Inc.), according to the manufacturer's instructions.

PRIMER DESIGN AND PCR

The PCR primers used to amplify the *SMN1*/*SMN2* genes are shown in Table 1 of the Supplemental Data that accompanies the online version of this article at <http://www.clinchem.org/content/vol52/issue3/>. Genomic DNA template (100 ng) was amplified in a total volume of 25 μL containing 0.12 μM each of the primers, 100 μM each

dNTP, 0.5 U of AmpliTaq Gold enzyme (PE Applied Biosystems), and 2.5 μL of GeneAmp 10 \times buffer II (10 mM Tris-HCl, pH 8.3; 50 mM KCl) in 2 mM MgCl_2 , as supplied by the manufacturer. PCR amplification was performed with an initial denaturation step at 95 $^\circ\text{C}$ for 10 min, followed by 35 cycles of 94 $^\circ\text{C}$ for 30 s, 53 $^\circ\text{C}$ for 45 s, and 72 $^\circ\text{C}$ for 45 s, with a final extension step of 72 $^\circ\text{C}$ for 10 min. PCR amplification was performed in a multiblock system (MBS) thermocycler (ThermoHybaid).

MULTIPLEX COMPETITIVE PCR

We used multiplex competitive PCR to amplify the *SMN1*, *SMN2*, *CYBB*, and *KRIT1* genes with the primers listed in Table 1 of the online Data Supplement. *KRIT1* and X-linked *CYBB* were used as internal controls for determining the absolute gene dosages of *SMN1* and *SMN2*. Multiplex competitive PCR was carried out in final volume of 50 μL containing 100 ng of genomic DNA, 0.02 μM each of the *CYBB* primers, 0.04 μM each of the *KRIT1* primers, 0.2 μM each of the *SMN* primers, 200 μM each of the dNTPs, 0.5 U of AmpliTaq Gold enzyme (PE Applied Biosystems), and 2.5 μL of GeneAmp 10 \times buffer II (10 mM Tris-HCl, pH 8.3; 50 mM KCl) in 2 mM MgCl_2 , as provided by the manufacturer. Amplification was performed in an MBS thermocycler with an initial denaturation step at 95 $^\circ\text{C}$ for 10 min, followed by 26 cycles of 94 $^\circ\text{C}$ for 30 s, 53 $^\circ\text{C}$ for 45 s, and 72 $^\circ\text{C}$ for 45 s, with a final extension step at 72 $^\circ\text{C}$ for 10 min.

CAPILLARY ELECTROPHORESIS

The HDA system with a GCK-5000 Cartridge Kit provided by eGene was used to analyze multiplex competitive PCR products to determine the gene dosage. PCR products were diluted 20-fold with deionized water and placed in the instrument sample tray. The DNA samples were automatically injected into the capillary channel and subjected to electrophoresis according to the eGene operation protocol. BioCalculator software (eGene) labeled the integrated peak area automatically.

PURIFICATION OF PCR PRODUCTS

For the primer extension reaction, the Microcon YM-100 system (Millipore) was used to remove excess primers and unincorporated dNTPs to purify the PCR products. The PCR products were quantified by use of a GeneQuant pro spectrophotometer (Amersham Pharmacia Biotech), and the samples were diluted to a final concentration of 10 ng/ μL for further experiments.

PinPoint ASSAY

The primer used to detect the critical C-to-T substitution by primer extension is listed in Table 1 of the online Data Supplement. Primer extension reactions were carried out in a 20- μL volume containing 50 ng of input PCR product and 0.75 μM primer, 25 μM each of the dideoxynucleoside triphosphates (Amersham Pharmacia Biotech), and 0.5 U of ThermoSequenase DNA polymerase (Amersham Bio-

sciences) in 1 \times supplied reaction buffer. The reaction was carried out in an MBS thermocycler. Each reaction was subjected to initial denaturation step at 96 $^\circ\text{C}$ for 60 s, followed by 50 cycles of denaturation at 96 $^\circ\text{C}$ for 15 s, primer annealing at 43 $^\circ\text{C}$ for 15 s, and extension at 60 $^\circ\text{C}$ for 100 s.

SAMPLE PURIFICATION FOR MALDI-TOF MS

The primer-extension products were dried under reduced pressure and then desalted by use of sequential POROS and cation-exchange chromatography. The DNA was purified by use of POROS 50-R2 (PerSeptive Biosystems) reversed-phase chromatography medium (23). Pipette tips were filled with 20 μL of POROS (80 g/L in 300 mL/L ethanol), the ethanol solution was drained out, and then 20 μL of 100 mmol/L triethylammonium acetate (TEAA, pH 6.5) was added to the tip. The DNA sample was extracted in the presence of 100 mmol/L TEAA (pH 6.5), rinsed sequentially with 100 mmol/L and 10 mmol/L TEAA (pH 6.5), and eluted with 200 mL/L acetonitrile. Solvent was removed by applying compressed air to the tip after all steps (24). Finally, the sample was dried in an SPD111V SpeedVac (Savant).

The sample was redissolved in 3–5 μL of water and desalted by cation-exchange chromatography with 5WX8-200 Dowex cation-exchange resin (Supelco Park) according to the protocol of Harksen et al. (25). The cation-exchange beads were first exchanged with 1 mol/L ammonium acetate and placed on a piece of Parafilm. DNA sample (5–10 μL) and an equal amount of matrix (see the section on *MS Analysis*) were added to 0.1 mg of beads and mixed up and down in the pipette tip several times. The supernatant was loaded on the sample plate for MALDI analysis.

MS ANALYSIS

Before MALDI-TOF MS analysis, the sample was mixed with matrix solution (50 g/L 3-hydroxypicolinic acid in a 4:5:1 mixture of water–acetonitrile–50 g/L diammonium citrate) and spotted on a 96 \times 2-well Teflon sample plate (PerSeptive Biosystems). MALDI-TOF mass spectra were acquired by a reflectron time-of-flight mass spectrometer (Voyager-DE PRO; Applied Biosystems). The instrument was equipped with a 337-nm nitrogen laser source at 3–20 Hz. Measurements were taken in linear, negative-ion mode at a 20-kV acceleration voltage and 380 ns delayed ion extraction. The excess unextended oligonucleotide primers were used as internal standards for mass calibration. A typical mass spectrum was obtained by averaging 100 laser shots followed by noise reduction and gaussian smoothing using Data Explorer software (Applied Biosystems).

Results

ANALYTICAL SCHEME

As shown in Fig. 1, the analytical scheme combines 2 complementary assays: (a) determination of the total copy numbers of *SMN* genes by multiplex competitive PCR

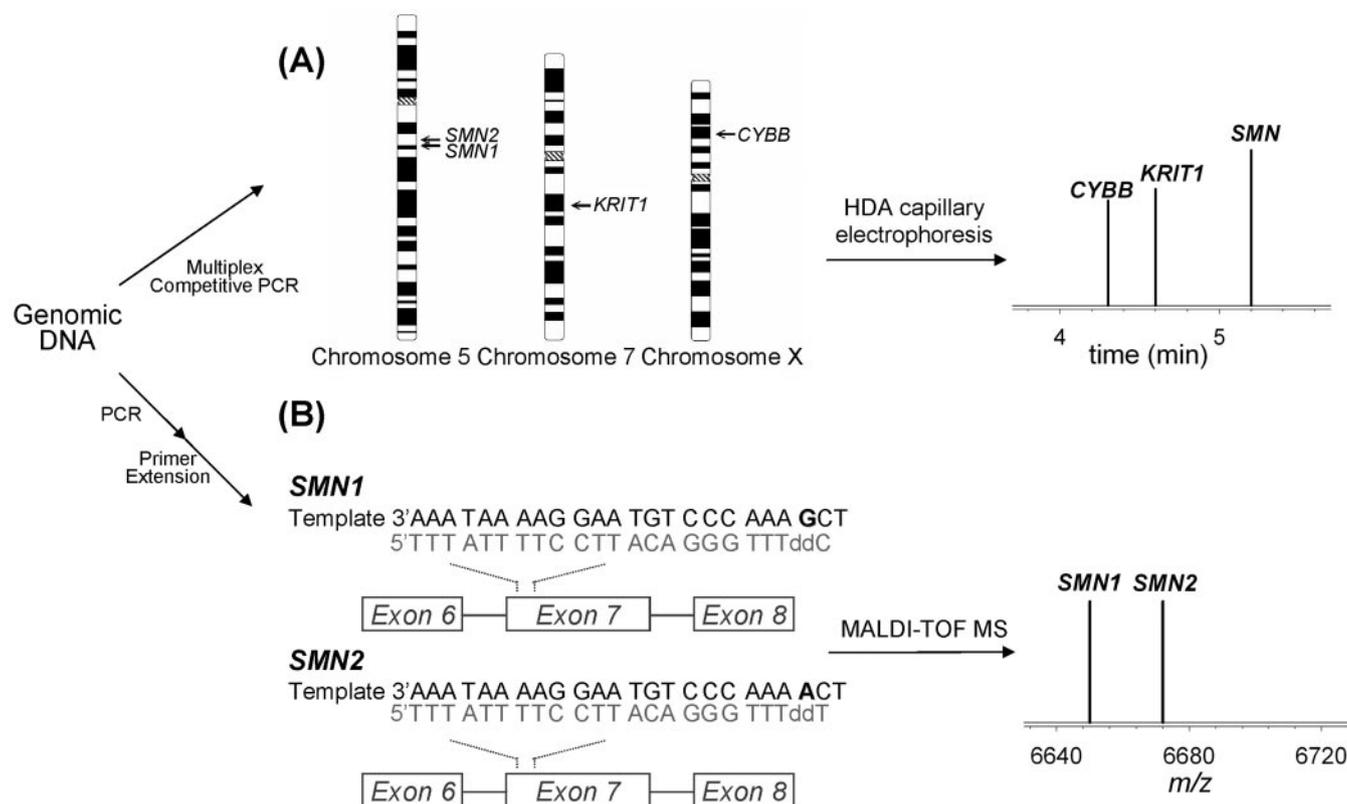


Fig. 1. Analytical scheme for quantitative genotyping of *SMN1* and *SMN2*.

(A), determination of absolute *SMN* gene dosage by capillary electrophoresis-based HDA. U, unknown sample; C, control sample. (B), determination of relative *SMN1* and *SMN2* gene dosages by MALDI-TOF MS.

and the capillary electrophoresis-based HDA system; and (b) determination of the relative ratio of *SMN1* and *SMN2* by PinPoint minisequencing and MALDI-TOF MS. We first determined the absolute gene dosages (sum of *SMN1* and *SMN2*), using *CYBB* and *KRIT1* as internal quantification references (Fig. 1A). After multiplex competitive PCR amplification, the PCR products were directly analyzed by the HDA system without further purification. The copy numbers of the *SMN* genes in the unknown samples (U) in comparison with the control samples (C) were calculated by use of Eqs. 1 and 2 below:

$$\text{Copy number} = \frac{\frac{\text{Peak area of SMN (U)}}{[\text{Peak area of CYBB (U)} \times K \text{ (U)}]}}{\frac{\text{Peak area of SMN (C)}}{[\text{Peak area of CYBB (C)} \times K \text{ (C)}]}} \times 4 \quad (1)$$

$$\text{Copy number} = \frac{\frac{\text{Peak area of SMN (U)}}{\text{Peak area of KRIT1 (U)}}}{\frac{\text{Peak area of SMN (C)}}{\text{Peak area of KRIT1 (C)}}} \times 4 \quad (2)$$

Because *CYBB* is X-linked, $K \text{ (U)}$ and $K \text{ (C)}$ represent the following factors: male = 2 and female = 1. Both *CYBB* and *KRIT1* were used for calculations, and the results were averaged.

To further determine the copy numbers of *SMN1* and

SMN2, we determined the relative ratio of *SMN1* and *SMN2* by PinPoint minisequencing and MALDI-TOF MS analysis (Fig. 1B). After sequential desalting with POROS and cation-exchange chromatography, the minisequencing products of the *SMN1* and *SMN2* alleles were subjected to MALDI-TOF MS analysis. The intensity ratio resulting from the C/T target-specific minisequencing products reflects the relative quantities of *SMN1* and *SMN2*. From a quantitative analysis standpoint, the different gene products in the PinPoint assay have similar masses to circumvent problems derived from distinct ionization efficiency. The relative ratio of *SMN1* to *SMN2* can be determined by Eq. 3:

$$\text{Ratio of copy number} = \frac{\text{Intensity of SMN1}}{\text{Intensity of SMN2}} \quad (3)$$

The combination of the 2 methods enables unambiguous determination of the individual copy numbers of *SMN1* and *SMN2* for the diagnosis of SMA.

DETERMINATION OF *SMN* COPY NUMBER BY CAPILLARY ELECTROPHORESIS

To quantify *SMN* copy number, we used a specific multiplex competitive PCR using primer pairs to amplify *CYBB* (X-linked), *KRIT1* (on chromosome arm 7q), and *SMN/SMN2* (as described above). *CYBB* and *KRIT1*, with

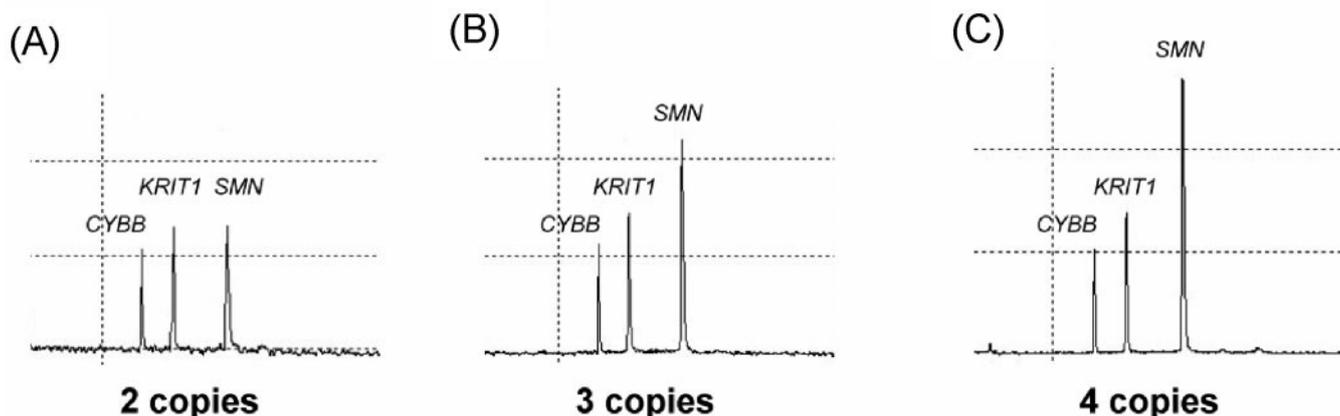


Fig. 2. Results of capillary electrophoresis-based HDA for individuals with 2 (A), 3 (B), and 4 (C) copies of the *SMN* genes.

known dosages, were used as genomic references to calculate the absolute gene dosages of *SMN1* + *SMN2*, collectively. Multiplex competitive PCR is based on the concept that the studied gene and its genomic references have equivalent PCR amplification efficiencies. Typical electrophoresis spectra of multiplex competitive PCR followed by detection with capillary electrophoresis are shown in Fig. 2. The spectra clearly show the different patterns of copy numbers 2, 3, and 4 for 3 male individuals (confirmed by quantitative real-time PCR). The fragments are well separated from each other; thus, the area of each peak can be used to calculate the total copy number of *SMN1* + *SMN2* in an unknown sample by use of Eqs. 1 and 2.

GENOTYPING AND QUANTIFICATION BY MALDI-TOF MS

In the second stage of our platform, the of *SMN1*/*SMN2* ratio was determined by minisequencing and MALDI-TOF MS. Typical mass spectra obtained from homozy-

gotes and heterozygotes (with different *SMN1*/*SMN2* ratios) are shown in Fig. 3. Mass differences of 15 were observed between primer extension products derived from the wild-type (i.e., *SMN1*) or mutant allele (i.e., *SMN2*), which unambiguously identified the sequence difference, C or T, at the polymorphic site. The measured gene ratios (*SMN1*/*SMN2*) were consistent with previous diagnoses made by quantitative real-time PCR analysis (data not shown). Quantification was performed by determining the ratio of the corrected peak height in Eq. 3, after baseline subtraction and gaussian smoothing, between minisequencing products of *SMN1* and *SMN2* in each MALDI mass spectrum.

For MALDI-TOF MS quantification of the *SMN1*/*SMN2* ratio, the between-run CV was 15% ($n = 6$). To further assess the accuracy of the quantitative method, we also evaluated the fluctuation between different positions on the MALDI sample plate. To minimize the spectral noise and spot-to-spot fluctuations in MALDI-TOF MS,

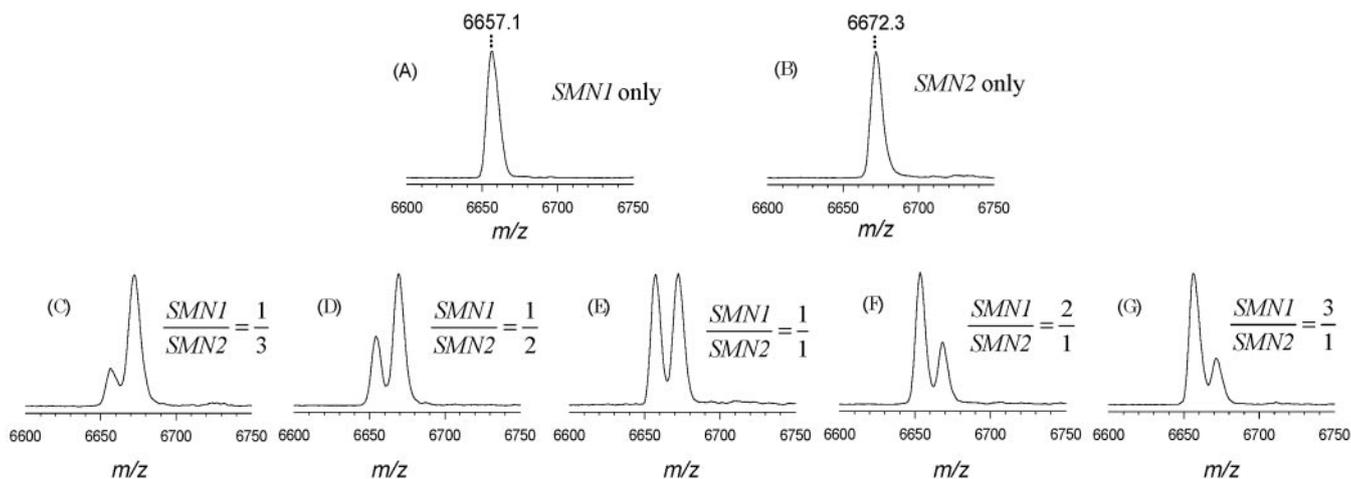


Fig. 3. MALDI-TOF MS analysis to detect minisequencing products with different *SMN1*/*SMN2* ratios from SMA patients and carriers, and healthy controls.

(A), individual with an *SMN1* gene only; (B), individual with an *SMN2* gene only; (C), individual with an *SMN1*/*SMN2* gene ratio of 1:3; (D), individual with an *SMN1*/*SMN2* ratio of 1:2; (E), individual with an *SMN1*/*SMN2* ratio of 1:1; (F), individual with an *SMN1*/*SMN2* ratio of 2:1; (G), individual with an *SMN1*/*SMN2* ratio of 3:1.

Table 1. Statistical evaluation of the measured *SMN* gene copy numbers determined by capillary electrophoresis.

<i>SMN</i> absolute copy number	Mean (SD) measured absolute copy number	No. of individuals	Frequency, %
4	3.90 (0.14)	320 ^a +9 ^b	64
3	2.87 (0.19)	170 ^a +9 ^b	34
2	2.09 (0.02)	10 ^a +2 ^b	2

^a Original number of cases from the general population.

^b Number of cases, including the patients and their families.

we used the mean of 5 replicate spectra to calculate the peak-area ratio. The CV from individual MALDI spots was <2% (n = 5). We evaluated the contribution of interference attributable to partial resolution in mass spectra to the quantification error based on peak intensity by deconvolution of the *SMN1*/*SMN2* genotype peaks, using gaussian peak fitting in the Origin 7.0 program (see Fig. 1 of the online Data Supplement). The difference was <5% of the measured peak height ratio. Experimental bias from instrument or sample handling therefore did not influence determination of the *SMN1*/*SMN2* ratio.

GENOTYPING OF SMA CARRIERS AND SMA-AFFECTED PATIENTS

Finally, to validate the suitability of our quantitative genotyping platform based on capillary electrophoresis and MALDI-TOF MS for high-throughput screening, we

analyzed 520 DNA specimens, including 478 samples from apparently healthy individuals from the general population, 9 from SMA-affected patients, and 33 from SMA carriers. In the first stage, the copy number of each genotype was determined by the HDA system (see Table 1 and Fig. 2 of the online Data Supplement). Calculation of the ratios of the peak areas *SMN*/*CYBB* or *SMN*/*KRIT1* gave mean (SD) *SMN* copy numbers for each genotype of 3.90 (0.14) for 4 copies, 2.87 (0.19) for 3 copies, and 2.09 (0.02) for 2 copies. In the second stage, we used all 520 DNA specimens from SMA patients, carriers from families of affected patients, and individuals from the general population to further evaluate the ability of the *SMN1*/*SMN2* ratio to identify SMA-affected individuals. Representative mass spectra for different *SMN1*/*SMN2* ratios are shown in Fig. 4. The statistical data indicate that the CV from individuals is between 4% and 10%, demonstrating that use of the PinPoint assay combined with MALDI MS can be a suitable method for quantification of the *SMN1*/*SMN2* ratio. In combination with the total copy number determined by the HDA system, the method unambiguously determined the genotypes of the 520 persons studied (Table 2). Consequently, the diagnostic accuracy of our method for detecting SMA is 100%.

The genotype distributions in the group of apparently healthy individuals, carriers, and SMA-affected patients derived from the MALDI-TOF MS and capillary electro-

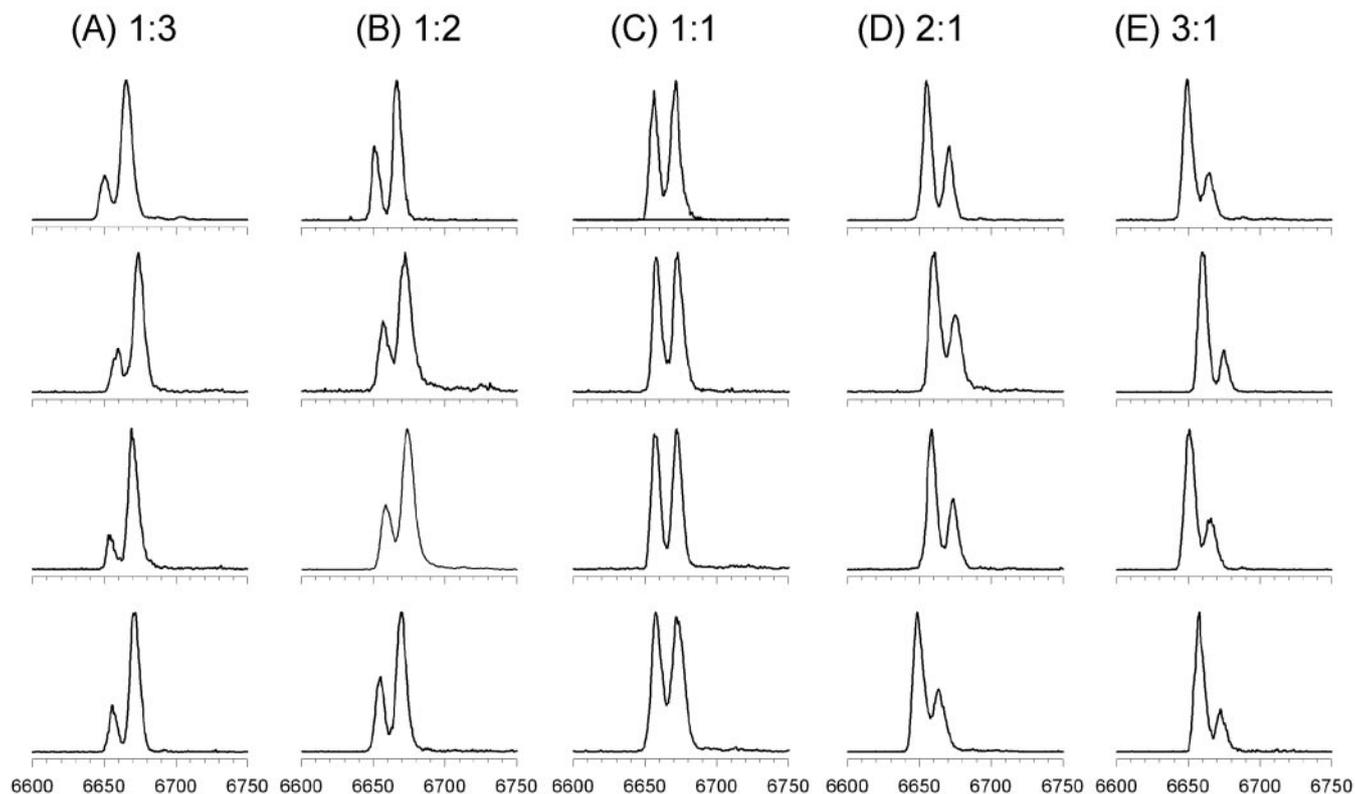


Fig. 4. MALDI-TOF MS analysis for different genotypes.

(A), *SMN1*/*SMN2* = 1:3; (B), *SMN1*/*SMN2* = 1:2; (C), *SMN1*/*SMN2* = 1:1; (D), *SMN1*/*SMN2* = 2:1; (E), *SMN1*/*SMN2* = 3:1.

Table 2. Summary of *SMN1/SMN2* genotype in 520 individuals, as determined by capillary electrophoresis and MALDI-TOF MS.

<i>SMN1/SMN2</i> genotype	Interpretation	<i>SMN1/SMN2</i> result			Predicted status
		Mean (SD)	CV, %	No. of individuals	
1/2	0.5	0.49 (0.02)	4.1	23	SMA carrier
1/3	0.33	0.32 (0.03)	9.0	10	SMA carrier
2/1	2	2.00 (0.13)	6.5	144	Normal
2/2	1	0.99 (0.06)	6.1	283	Normal
3/1	3	3.19 (0.30)	9.4	21	Normal
<i>SMN1</i> only	∞	No <i>SMN2</i> peak		30	Normal
<i>SMN2</i> only	0	No <i>SMN1</i> peak		9	SMA patient
Total				520	

phoresis-based HDA analyses are shown in Fig. 5. Among the healthy individuals, 437 of 478 had 2 *SMN1* copies (*SMN1/SMN2* ratios of 2:2, 2:1, or 2:0), 31 of 478 carried 3 *SMN1* copies (*SMN1/SMN2* ratios of 3:1 or 3:0), and the remaining 10 carried 4 *SMN1* copies (*SMN1/SMN2* ratio of 4:0). Among the SMA patients, 5 of 9 had 4 *SMN2* copies (*SMN1/SMN2* ratio of 0:4), 2 of 9 had 3 *SMN2* copies (*SMN1/SMN2* ratio of 0:3), and 2 of 9 had 2 *SMN2* copies (*SMN1/SMN2* ratio of 0:2). To confirm the *SMN* gene dosages, we performed quantitative real-time PCR, using TaqMan technology with minor groove binder probes (26), and a specific multiplex competitive PCR protocol coupled with denaturing HPLC (data not shown) (27). The excellent agreement demonstrated that our approach is capable of making an unambiguous distinction between carriers and noncarriers.

Discussion

SMA is one of the most frequent severe autosomal recessive muscular diseases, with a birth prevalence of 1 in 10 000. The absence of both copies of the *SMN1* gene is the most common mutation type of this disease and accounts for 94% of all SMA alleles in this population. Because of the high carrier frequency of 1 in 35 to 1 in 50 in the

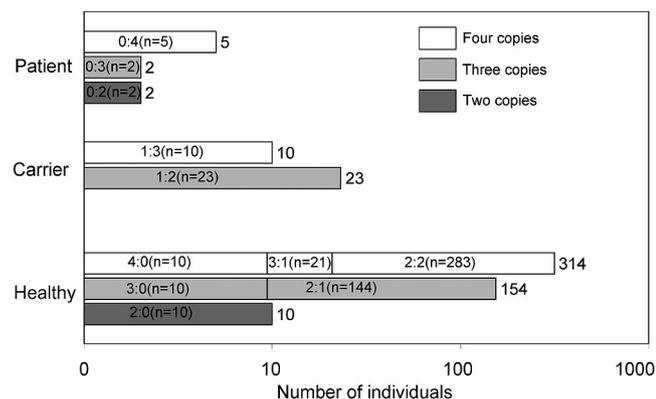


Fig. 5. Distribution of copy numbers among apparently healthy individuals, SMA carriers, and SMA-affected patients.

The *SMN1/SMN2* ratios are indicated inside the bars.

worldwide population (1, 2), prenatal diagnosis and carrier screening of SMA are currently the best solutions to minimize the incidence of SMA. Various assays have been used for the screening and diagnosis of SMA. Several different quantitative PCR tests for *SMN* analysis have been developed (3, 10, 26, 28, 29). Although these methods efficiently determine *SMN1* and *SMN2* gene copy numbers, most of the methods are based on fluorescent labels or hybridization assays, which are often susceptible to complications attributable to nucleic acid secondary structure (30, 31). Heteroduplex formation may affect quantitative PCR–restriction fragment length polymorphism analyses (32). Heteroduplexes between *SMN1* and *SMN2* PCR products cannot be digested by *DraI*, an enzyme that is commonly used in the restriction fragment length polymorphism analyses, which increases the *SMN1* signal while decreasing the *SMN2* signal (33).

Our parallel assays for screening SMA carriers and patients determine both the *SMN1/SMN2* ratio and the absolute *SMN* gene dosage by 2 independent methods. Our results demonstrate that the complementary assays together create a rapid and reliable method for quantifying *SMN1/SMN2* genes. In an analysis of 520 genomic DNA samples of known genotype from healthy individuals, carriers, and SMA patients with homozygous deletions or conversions, the quantification platform had 100% assay specificity and sensitivity. In all cases, the carriers had only 1 *SMN1* gene; because of the small size of our screening sample, no carriers with 1 *SMN1* and 1 *SMN2* gene or 1 *SMN1* and no *SMN2* gene were observed. In the case of *SMN2*, the *SMN2* copy number of affected patients was related to the phenotype (data not shown). It should be noted that, like all other previously described assays, our assay does not detect the remaining mutations. Therefore, patients who are compound heterozygotes for a common point mutation will not be identified by the current method. For affected patients who are genotyped as having 1 *SMN1* gene, it might be prudent to genotype the *SMN1* gene to determine the possibility of compound heterozygosity with a point mutation allele.

Current capillary electrophoresis instruments use lasers as the light source for DNA analysis, which is bulky and expensive. By combining HDA with multiplex competitive PCR, we can identify *SMN* gene dosage based on the peak area compared with known genes. There was no loss of resolution with the LED light source for detecting multiplex competitive PCR products. Additionally, capillary electrophoresis was optimized for rapid screening of multiplex PCR products. In our study, automated capillary electrophoresis of multiplex competitive PCR products can be completed with the HDA system within 10 min. For high-throughput analyses, up to 96 DNA samples can be analyzed within 1.5 h.

Primer extension combined with MALDI MS is a direct method for detecting SNPs. Mass can be measured accurately to determine the exact nucleotide at the mutation site without the need for additional confirmation. Detec-

tion specificity is therefore better than can be achieved with fluorescence-based genotyping. Another advantage of this combination of techniques is its high-throughput capacity to screen large populations. In our improved MALDI-TOF MS analysis platform, sequential desalting can be completed within a few minutes and is amenable to automation, thereby allowing adaptation for robust high-throughput SNP genotyping.

The development of new molecular tools will favor a viable and cost-effective detection method. We calculated the costs of this assay at approximately \$5.00 (US) per sample. Excluding the instruments, this cost includes chemicals, reagents, and primers for PCR amplification, and reagents and supplies for purification, primer extension, capillary electrophoresis-based HDA, and MALDI-TOF MS. The assay may be most cost-effective in clinical laboratories with available equipment. Furthermore, the automated HDA system and MALDI-TOF MS can provide a rapid assay for molecular diagnostic. The strategy described here can be adapted to characterize individual genotypes with respect to other disease-causing mutations; as such, it may be widely applicable in the clinical setting for diagnostic genotyping.

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