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ARTICLE

# SMA carrier testing – validation of hemizygous SMN exon 7 deletion test for the identification of proximal spinal muscular atrophy carriers and patients with a single allele deletion

Hans Scheffer, Jan Maarten Cobben, Rob GJ Mensink, Rein P Stulp, Gerrit van der Steege and Charles HCM Buys

*Department of Medical Genetics, University of Groningen, The Netherlands*

**To facilitate the detection of carriers of a hemizygous survival motor neuron (*SMN*) exon 7 deletion we have modified the quantitative *SMN* exon 7 assay described by McAndrew *et al* (1997). The major changes include quantitative analysis of the amount of *SMN* exon 7-specific fluorescently-labelled PCR product on an automated sequencer, and the monitoring of the completeness of a *DraI* digestion necessary to distinguish the PCR products of exons 7 of *SMN* and its copy gene. In our method the amount of *SMN* exon 7 PCR product is compared with the amount of a co-amplified PCR product of the retinoblastoma (*RB1*) exon containing a *DraI* restriction site. By co-amplification using the same primers of plasmids included in the reaction as internal standards containing *SMN* exon 7 with a 36-nucleotide deletion and *RB1* exon 13 with a 19-nucleotide deletion, respectively, the relative amplification efficacy can be monitored. The assay has been validated in 63 ascertained carriers and 28 ascertained non-carriers. The sensitivity of the test is approximately 97%, the specificity approaches 100%. In four out of six SMA patients without a homozygous deletion we detected a hemizygous deletion. The implications of the use of this assay for carrier testing and for confirmation of the clinical diagnosis of SMA are discussed.**
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**Keywords:** spinal muscular atrophy; SMA; carrier testing; survival motor neuron; *SMN*

## Introduction

With a prevalence of about 1/10 000,[1,2] and a carrier frequency of 1/40–1/60[3,4] the proximal spinal muscular atrophies (SMAs) are among the most frequent autosomal recessive hereditary disorders. Patients can be classified clinically into three groups: acute, intermediate, and mild (SMA types I, II, and III,respectively).[5,6] The SMA 5q13 region shows a complex structure at the genomic level, including a 500-bp duplication and inversion.[7] The survival motor neuron (*SMN*) gene is present in at least one telomeric (*SMN1*) and one centromeric copy (*SMN2*) per chromosome in normal (non-carrier) individuals.[7] The two *SMN* genes (*SMN1* and *SMN2*) are highly homologous and contain only five base-pair differences within their 3' ends.[8] The differences in exons 7 and 8 are used to distinguish *SMN1* and *SMN2* in direct diagnosis of SMA. Deletions of the *SMN1* gene located at chromosome 5q13 appear to be directly involved in SMA,[7] since exon(s) 7 (and 8) of *SMN1* are undetectable in over 95% of patients, irrespective of their clinical type,[7,9–12] either as a result of homozygous deletions, or because of conversion of sequences of *SMN1* to those in the *SMN2* gene.[7,11–16] The identification of several small mutations in the *SMN1* gene in patients without a deleted or sequence-converted *SMN1* allele provides strong evidence that this gene is the primary SMA-determining gene.[7,8,17–25] The clinical phenotype of SMA appears to be determined by the underlying mutational mechanism. Most cases of SMA type I result from homozygous gene-deletion events, whereas the

**Correspondence: H Scheffer, Department of Medical Genetics, University of Groningen, Antonius Deusinglaan 4, NL-9713 AW Groningen, The Netherlands. Tel: +31 50 3632925; Fax: +31 50 3632947; E-mail: h.scheffer@med.rug.nl**
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milder forms of the disease, SMA types II and III, result mostly from gene conversion of *SMN1* to *SMN2*, in SMA type II a gene conversion event in one allele in addition to a hemizygous deletion event in the other allele, or gene conversions in both alleles in SMA type III.[13,15,16,26,27] As a result, patients suffering from the milder forms of SMA carry more copies of the *SMN2* gene.

The absence of SMN1 exon(s) 7 (and 8) in SMA patients can be used as a diagnostic tool for SMA,[28] with a sensitivity of approximately 95% in clinically strictly defined SMA patients. This assay is not quantitative, however, and the remaining patients, whom based on Hardy-Weinberg equilibrium are virtually all assumed to have a hemizygous *SMN1* absence of at least exon 7, either by deletion or by conversion. McAndrew *et al*[21] have described a quantitative assay based on competitive amplification of *SMN1* and *SMN2* exons 7 to determine their gene-copy number. We have modified their approach by 1) substitution of the genomic reference amplicon containing no *DraI* restriction site (an exon from the cystic fibrosis conductance transmembrane regulator (*CFTR*) gene) by an amplicon that does contain a *DraI* restriction site, notably exon 13 from the retinoblastoma (*RB1*) gene, allowing to monitor the completeness of *DraI* digestion necessary to distinguish exon 7 of *SMN1* and *SMN2*;[28] and by 2) addition of a fluorescent end label to one of the primers of each amplicon, allowing the use of an automated sequencer for fragment separation and quantification. Furthermore, we have validated this assay by analysing its outcome in a cohort of ascertained carriers (parents of multiple affected children) and non-carriers (siblings of SMA patients with two non-risk SMN alleles based on haplotype analysis of flanking micosatellite markers). The occurrence of two *SMN1* genes on a single chromosome 5, as observed by McAndrew *et al*[21] and Wirth *et al*[29] would bear on the sensitivity of this technique for carrier identification, since individuals with an *SMN1* [1.1] genotype (non-carriers) cannot be distinguished from individuals with an *SMN1* [0.2] genotype (carriers). Since in clinical DNA service practice the majority of the tests used are currently based on qualitative analyses, we felt that implementation of a quantitative assay should be preceded by a thorough validation of sensitivity and specificity. In principle, this method can also be used to determine the *SMN2* exon 7 copy number.

## Patients, materials and methods

### SMA patients and DNA isolation

Clinical assessment of the SMA patients has been described elsewhere.[10] The diagnosis of SMA was in agreement with the strict criteria established by the International SMA Consortium.[30] As ascertained carriers, 63 parents of multiple affected children with a homozygous SMN exon 7 deletion have been investigated. As likely non-carriers, 31 siblings of SMA patients with two non-risk 5q haplotypes as shown by linkage analysis using flanking informative markers have been studied. DNA was isolated from peripheral blood by the salting out method,[31] or from chorionic villus samples by organic extraction.[32]

### Synthesis of internal standards

Two internal standards (IS) were constructed for standardisation and monitoring of the amplification efficiency of both competitive PCR reactions in the *SMN1* copy number assay (see Figure 1) by incorporation of equimolar amounts of the two ISs. The SMN-IS has an internal deletion of 36 bp. The *RB* exon 13 IS (RB-IS) has an internal deletion of 19 bp. Both ISs can be amplified by the same primers as their genomic counterparts
(in the case of *SMN* two genomic counterparts, notably *SMN2* and *SMN1*, distinguished by *DraI* digestion), but can be separated by size. *RB-IS* contains a *DraI* site, and therefore the completeness of *DraI* digestion can be monitored. Genomic DNA was used as a template to generate both ISs. A 25 μl reaction contained 100 ng genomic DNA, 3 mM $MgCl_2$, 200 μM each dNTP, 20 ng each of R111 (5'-AGACTATCAACTTAATTTCTGATCA-3') and SMN7IS (TCGATAGATACAGATATATCGATATCTGTTTTAGTTTTTCTTC CTTCC-3') primers for SMN-IS and 20 ng each of RBex13F (5'-ATTACACAGTATCCTCGACA-3') and RBIS13 (5'-TATACGAACTGGAAAGATGCCTATAGTACCACGAATTACAATG-3') primers for RB-IS, and 0.5 U AmpliTaq polymerase (Perkin Elmer, Foster City, CA, USA). The reactions were denatured at 95°C for 5 min, then run for 33 cycles at 95°C 1 min, 55°C 2 min, and 72°C 3 min (Perkin Elmer 9600 thermocycler). The products were subcloned into pCR2.1 (Invitrogen, Carlshad, CA, USA), and the plasmids were purified according to standard procedures. As internal standards 15 000 genome equivalents of intact plasmids were used in the copy-number assay. Note that the SMN-IS does not contain a *DraI* site.

### SMN1 Copy-number assay

Two competitive amplifications are performed in a multiplex PCR reaction. The genomic DNA concentration was determined by spectrofluorometric analysis. Per reaction approximately 50 ng DNA was used (about 15 000 genome equivalents) in the presence of 3 μl of a mixture containing 5000 molecules/μl of SMN-IS and RB-IS each with 20 ng each of fluorescently-labelled R111 and non-labelled X7-Dra (5'-CCTTCCTTCTTTTTGATTTTG TTT-3'[28] primers for SMN products, and 20 ng each of RBex13F and fluorescently-labelled RBex13R (5'-TATACGAACTGGAAAGATGC-3') primers for RB products, respectively. The 25 μl reaction contained 3 mM $MgCl_2$, 200 μM each dNTP, and 0.5 U AmpliTaq polymerase (Perkin Elmer). The reactions were denatured at 95°C for 5 min, then run for 24 cycles at 95°C for 1 min, 55°C for 2 min, and 72°C for 3 min (Perkin Elmer 9600 thermocycler). The PCR products (15 μl) were digested with 10 U *DraI* overnight at 37°C. The digested samples were run on a 6% denaturing polyacrylamide gel on an automated sequencer

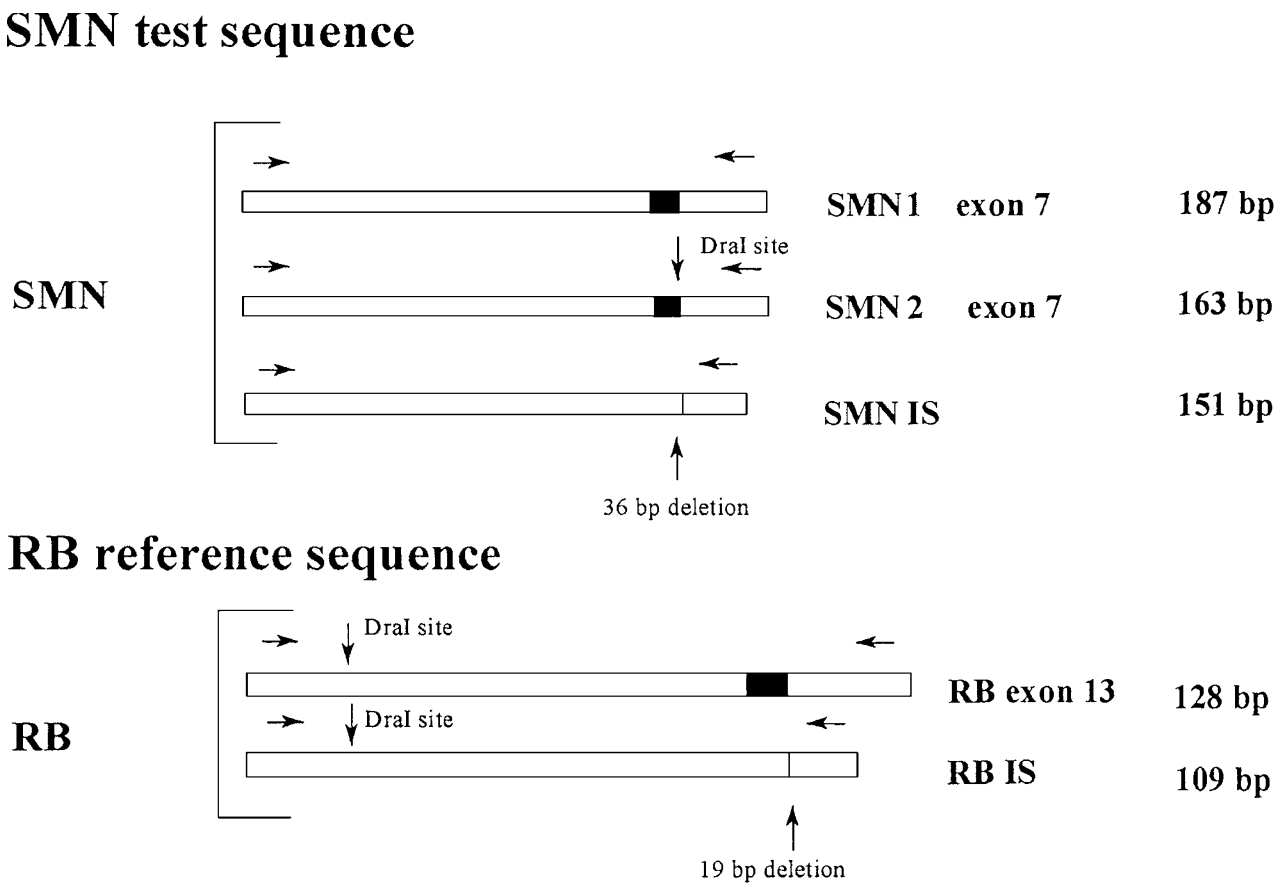


**Figure 1** Amplification strategy of the quantitative SMN exon 7 analysis. Deletion products of SMN exon 7 (36 bp deletion) and RB1 exon 13 (19 bp deletion) have been generated by PCR, and subcloned into pCR2.1. These plasmids have been added as internal standards (SMN-IS and RB-IS, respectively). Three PCR-products are generated by the SMN primers: SMN1, SMN2, and SMN-IS. SMN1 and SMN2 can be separated by DraI digestion. Two PCR-products are generated by the RB1 primers: RB-genomic, and RB-IS. The RB-genomic and the RB-IS contain a DraI restriction site, enabling the monitoring of the completeness of digestion. The forward SMN primer and the reverse RB primer are fluorescently end-labelled. The length of the PCR-products in base pairs is indicated.

(ALF, Pharmacia, Sweden) and the peak intensity was quantified by measuring the Area Under the Curve (AUC).

Completeness of *DraI* digestion was monitored by absence of undigested products of 237 bp (*RB* exon 13 product) and 218 bp (*RB-IS*). Since there are two copies of RB per genome, the genomic *SMN1*/genomic RB ratio was used to determine the relative copy number of *SMN1* for all samples, and corrected for differences in amplification efficiency of the two competitive PCR reactions by multiplication using a factor RB-IS/SMN-IS.[33] This corrected ratio is normalised for the mean of the ratios obtained in two standard reference samples (from individuals carrying two copies of *SMN1* exon 7) that were included in all gels. Also included in all gels were two reference samples from hemizygous *SMN1* exon 7 deletion carriers and a blank sample lacking genomic DNA. Non-carriers have a normalised and corrected ratio over 0.75; carriers have a normalised and corrected ratio under 0.75. All reactions were duplicated. Only those results have been taken into account of which the duplicate reaction results did not differ by more than 20%.

## Results

### Carriers

In 61 out of 63 samples from parents of multiple-affected children with SMA, a normalised and corrected *SMN1*/*RB* genomic ratio compatible with a single *SMN1* exon 7 copy was found. A representative analysis is shown in Figure 2. The average ratio observed in these 61 samples was 0.54 ± 0.12 (mean ± SD) ranging from 0.27 to 0.70. The values of the normalised and corrected *SMN1*/*RB* genomic ratio for these 61 carriers are shown in Figure 3. In two of 63 samples a normalised and corrected *SMN1*/*RB* genomic ratio compatible with two *SMN1* exon 7 copies was found (1.12 and 1.24; the two left-hand samples in Figure 3).

**Non-carriers**

In three samples the test results obtained were obviously unreliable (see Discussion). In 27 out of 28 samples from ascertained carriers, a normalised and corrected *SMN1/RB* genomic ratio compatible with two *SMN1* exon 7 copies was found. The average of this ratio observed in these 27 samples was 1.11 ± 0.25 (mean ± SD) ranging from 0.78 to 1.79. The values of the normalised and corrected *SMN1/RB* genomic ratio for these 27 non-carriers are shown in Figure 3. In one sample the *SMN1/RB* genomic ratio is 1.79, differing more than 2 SD from the average value of 1.11, most likely indicating that this sample may in fact contain three *SMN1* exon 7 copies. In one of 28 samples a normalised and corrected *SMN1/RB* genomic ratio compatible with a single *SMN1* exon 7 copy was found (0.28). This sample contained DNA isolated from a chorionic villus sample. After revaluation of a DNA sample isolated from leukocytes from the same meanwhile healthy born individual, we detected a normalised and corrected *SMN1/RB* ratio compatible with two *SMN1* copies.

**Patients**

Six patients with clinical confirmed proximal SMA without a homozygous *SMN* exon 7 deletion were analysed. In four of these six patients a normalised and corrected *SMN1/RB* genomic ratio compatible with a single *SMN1* exon 7 copy was found. A representative analysis is shown in Figure 4. The presence of a hemizygous deletion of *SMN1* exon 7 was confirmed in one parent in all four cases. In one case out of four hemizygous deleted SMA patients, we identified in the other allele a point mutation which creates a novel splice site in exon 4 of *SMN1* and thus disturbs the reading frame of the *SMN1* gene (submitted).[34] In two out of six patients, including one with a haploidentical healthy sibling, a normal *SMN* exon 7 intensity was detected.

## Discussion

**Technique**

Our method of determining the number of *SMN1* genes in a given DNA sample implies two improvements when compared with the original procedure described by McAndrew *et al*.[21] Because the DNA sequences are amplified with a fluorescently end-labelled primer in each amplicon, an automated sequencer can be used for fragment separation and quantification. In addition, we substituted an amplicon containing a *DraI* restriction site, namely exon 13 of the retinoblastoma (*RB1*) gene for the original genomic reference amplicon – an exon from the cystic fibrosis conductance transmembrane regulator (*CFTR*) gene, which contained no *DraI* restriction. This allows monitoring of the completeness of *DraI* digestion necessary to distinguish exon 7 of *SMN1*

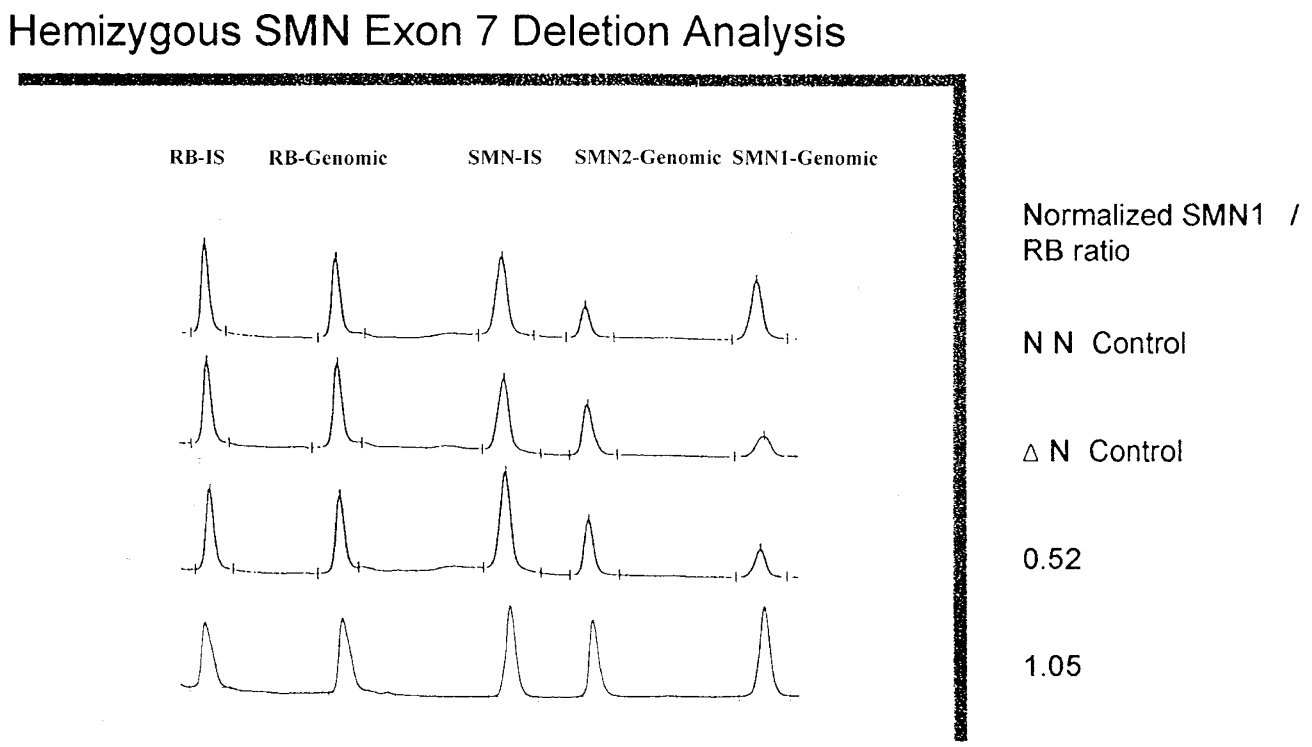


**Figure 2** Example of carrier detection by quantitative SMN exon 7 deletion analysis. The patterns by the automated sequencer obtained from an ascertained carrier (line 3) and an ascertained non-carrier (line 4) are compared with a normal reference sample (line 1) and a hemizygous deletion reference sample (line 2). The origin of the signals is indicated on top. The normalised SMN1/RB ratios are indicated on the right.

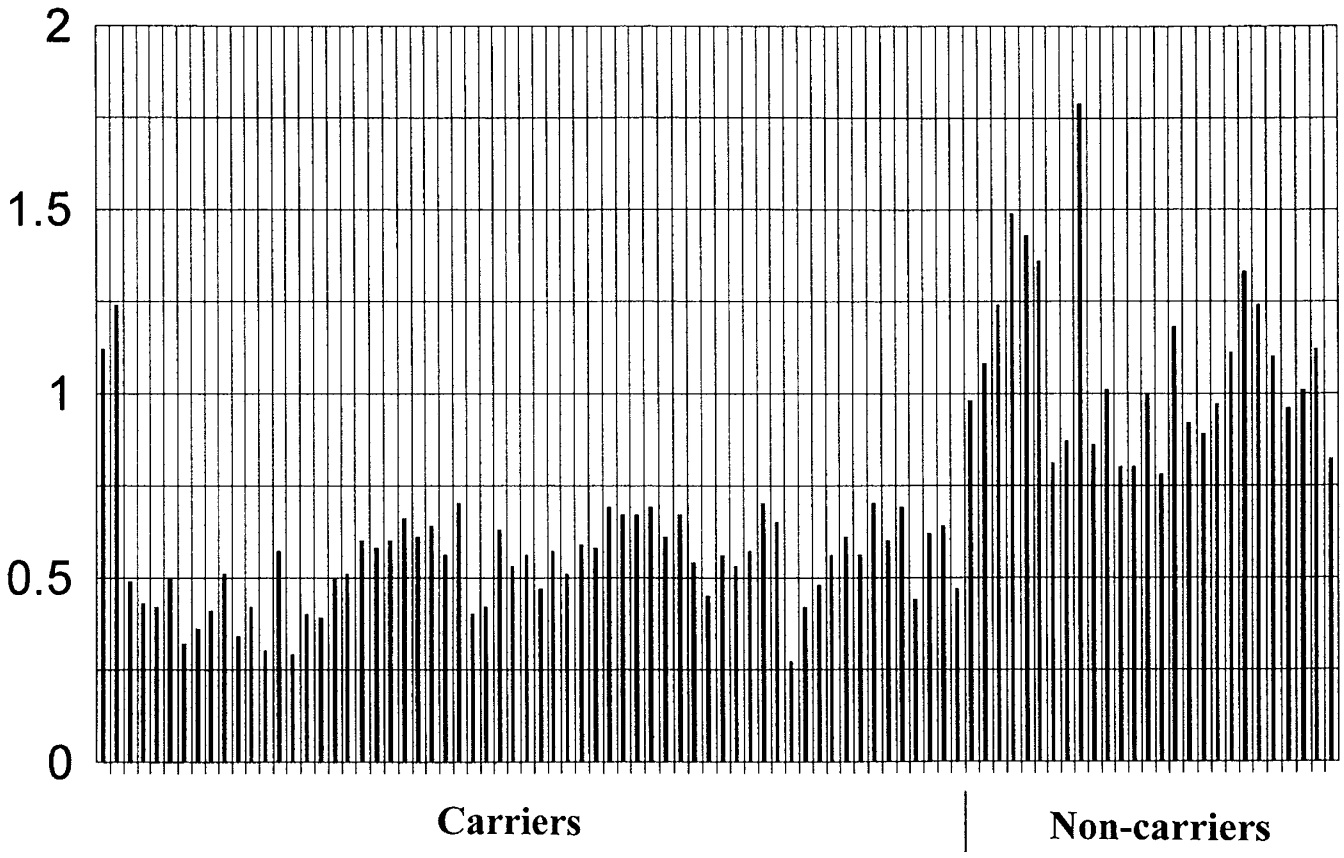


**Figure 3** Histogram of normalised SMN1/RB values in carriers and non-carriers.

from that of *SMN2*.[28] It may also be noted that rare individuals with an *RB1* deletion have a phenotype, ie retinoblastoma, and will therefore be recognised as *RB1* deletion carriers, whereas *CFTR* deletion carriers such as described by Morral *et al*[33] will not, since cystic fibrosis is a recessive disorder. Furthermore, we calculated a corrected *SMN1*/*RB* ratio based on the generally applicable quantitative PCR method described by Celi *et al*.[35] This calculation takes into account a possible difference in amplification efficiency for the two competitive PCR reactions used. We noted that reliable results are only obtained if the signals of the *RB-IS*, *SMN-IS*, and *RB*-genomic amplification products do not differ greatly. As a rule of thumb, the signal with the lowest intensity may not be less than 50% of the highest signal of the three PCR products. In Figure 3 it can be seen that no overlap in values of the *SMN1*/*RB* ratio for carriers and non-carriers was observed (except for the two samples of carriers showing *SMN1*/*RB* values consistent with two *SMN1* genes; see also Carriers). To verify whether gel deviations exist, the SD within each gel has also been calculated for carriers and non-carriers, and the resulting SDs were summed and divided by the number of gels. For carriers a mean SD per gel of ± 0.08 was calculated, which is considerably lower than the SD of ± 0.12 for the analysis as a whole. This was also observed for the non-carriers, where a mean SD per gel of ± 0.20 was calculated, compared with an SD of ± 0.25 for the analysis as a whole. This analysis using batch statistics indicates that for clinical use the most reliable results will be obtained by comparison of samples within a single gel. At least one known normal reference sample and one known carrier reference sample should be included per gel.

**Carriers**

The identification of two individuals with a double *SMN1* dose among 63 ascertained carriers can be explained in several ways. Theoretically, a germ line mosaicism for an *SMN1* exon 7 (and 8) deletion cannot be ruled out. The DNA samples originate from parents with multiple-affected offspring, which might be explained by the existence of more than one germ cell harbouring an *SMN1* exon 7 (and 8) deletion not present in DNA from leukocytes of these individuals. Case 1 is a father of multiple-affected children (false paternity highly unlikely based on data from microsatellite analysis in the family), whereas case 2 is a mother of multiple-affected children. However, since Wirth *et al*[36] have shown that *de novo* deletions in *SMN1* occur during meiotic cell divisions in the majority of cases, and germline mosaicism is thought to occur in mitotic cell divisions during early

development of germ cells in both spermatogenesis and oogenesis, we consider this possibility, although not fully excluded, to be at least as unlikely. A more likely explanation can be found in observations of the occurrence of two *SMN1* genes on one chromosome 5.[21,29] In their data sets, McAndrew *et al*[21] identified one out of 79 carriers and three out of 64 non-carriers and Wirth *et al*[29] identified four out of 115 individuals (carriers and non-carriers) with *SMN1* copies on a single chromosome. Taking these data sets and our data together, in 10 out of 321 (3.1%) individuals (carriers and non-carriers) two *SMN1* copies on a single chromosome have been identified. This phenomenon would bear on the sensitivity of this technique for carrier identification, since individuals with an *SMN1* [1.1] genotype (non-carriers) cannot be distinguished from individuals with an *SMN1* [0.2] genotype (carriers). From our data, we therefore calculate a sensitivity of approximately 97% for this technique in carrier detection amongst relatives of patients with an identified *SMN1* exon 7 (and 7) deletion. This is consistent with the sensitivity calculated from the three data sets taken together. For carrier detection among random individuals without a positive family history of SMA one can expect an additional drop in sensitivity, in view of the existence of mutant non-exon 7 (and 8) deletion *SMN1* alleles. From the fact that approximately 95% of SMA patients are homozygous for an *SMN1* exon 7 (and 8) deletion, we estimate, assuming Hardy-Weinberg equilibrium for all types of SMN mutations, that such mutant non-exon 7 (and 8) deletion *SMN1* alleles represent approximately 2.2% of all pathogenic *SMN1* alleles. Wirth *et al*[29] have shown that 3.4% of typical SMA patients show subtle mutations, all hemizygous for *SMN1* exon 7, and therefore mutant non-exon 7 (and 8) deleted *SMN1* alleles should represent approximately 1.7% of all pathogenic *SMN1* alleles. The practical consequence is that the sensitivity of this technique for carrier identification in random individuals is approximately 95.3% (97% – 1.7%).

**Non-carriers**

In three samples the tests were obviously unreliable because the duplicated reaction results differed by more than 20%. The identification of one individual with a single *SMN1* dose out of 28 ascertained non-carriers can also be explained in several ways. Theoretically, a double crossover between the markers used for initial linkage analysis in this family cannot be ruled out (likelihood less than 1%). However, since this case represents a DNA sample isolated from a chorionic villus sample, we considered it more likely that because of the difference in DNA isolation procedures used for leukocytes and chorionic villus samples, an incorrect ratio was determined, possibly due to degradation of genomic DNA during storage of the sample. We reanalysed DNA isolated from leukocytes from this meanwhile healthy born individual. A

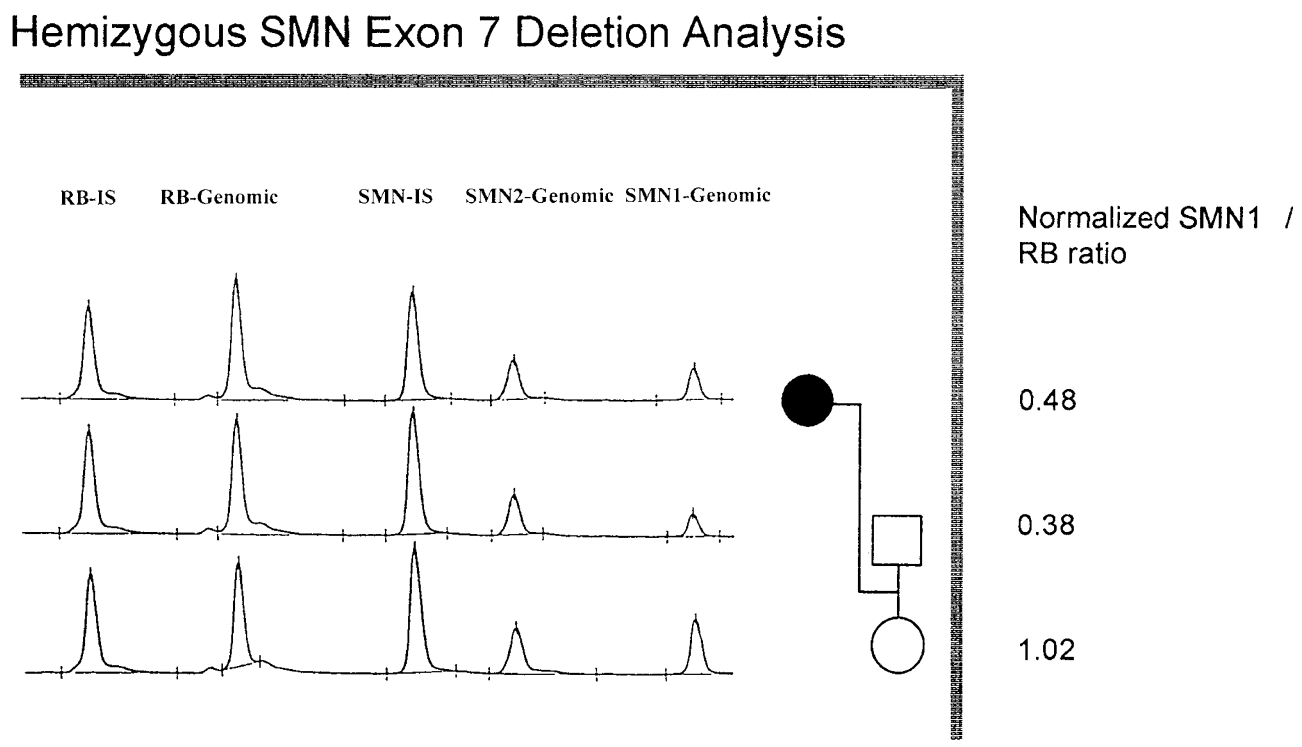


**Figure 4** Example of hemizygous SMN exon 7 deletion detection in an SMA family. The affected child is indicated by a solid symbol. The origin of the signals is indicated at the top. The normalised SMN1/RB ratio is indicated on the right. The normalised SMN1/RB ratios are indicated on the right. Note that ratios of approximately 0.5 indicative of a hemizygous SMN exon 7 deletion have been detected in the affected child and the carrier father, and a ratio of approximately 1.0 indicative of two SMN exons 7 in the mother.

normalised and corrected *SMN1*/*RB* genomic ratio compatible with a two-*SMN1* exon 7 copy was found, confirming that the *SMN1*/*RB* ratio was determined incorrectly due to the quality of the chorion villus DNA sample. We conclude from this finding that the specificity of the test after revaluation of the aberrant sample is close to 100% (95% confidence interval 87–100%). In conclusion, only DNA samples isolated by the same procedure can reliably be compared with reference samples using this technique. Samples of DNA isolated from chorionic villi cannot be stored prior to quantitative analysis, since endonucleases are apparently not rigorously inactivated by the extraction procedure used here.

### Consequences for risk calculations

In applications for carrier testing of relatives of an SMA index patient with an ascertained homozygous *SMN1* exon 7 (and 8) deletion, a sensitivity of 97% should be used. Assuming an *a priori* risk of 1/2 (uncles and aunts of patients), or 2/3 (healthy siblings of patients), and a specificity of the test of 100%, a non-carrier test result would lead to *a posteriori* risks of approximately 2.9% (uncles and aunts) and 5.7% (siblings), respectively. If a hemizygous deletion could be detected in parents of an SMA patient prior to a carrier test in an unaffected sibling, and the test result in this sibling were negative, then being a carrier would be virtually excluded.

For carrier testing of random individuals without positive family history of SMA assuming an *a priori* risk of 1/45 of being a carrier of any mutant *SMN1* allele and a specificity of the test of 100%, a test sensitivity of 95% should be used. If an *SMN1*/*RB* ratio compatible with two *SMN1* genes is found, the *a posteriori* risk of being a carrier is 0.11. If an *SMN1*/*RB* ratio compatible with one *SMN1* gene is found, the *a posteriori* risk of being a carrier is 99.89%.

### Patients

The two patients in whom we could not identify a hemizygous *SMN1* exon 7 deletion reflects the fact that proximal SMA-like diseases exist. We consider it less likely, however, that locus heterogeneity exists in true recessive proximal SMA, since non-5q-haploidentical affected sibs have never been identified. Given the current limited knowledge of *SMN* function the possibility of dominant mutant *SMN* alleles cannot be excluded.

Since 95% of proximal SMA patients have a homozygous *SMN1* exon 7 (and 8) deletion, this test may confirm the clinical diagnosis in the vast majority of cases. However, when no homozygous deletion is observed, the detection of a hemizygous deletion using the test described here will give a strong suggestion of confirmation of the clinical diagnosis in virtually all cases, since it follows from the Hardy-Weinberg equilibrium that less than 0.6% of patients will have a mutant non-deletion *SMN1* allele on both chromosomes. In case of a hemizygous deletion, it cannot be excluded, however, that the individual is merely a carrier of an *SMN1* exon 7 (and 8) deletion. It also follows that whenever no hemizygous deletion is found in a patient suspected on clinical grounds of having SMA, the diagnosis of 5q-linked SMA can be practically rejected. It is our experience that on scrutinising the clinical data, or on clinical revaluation of a patient without a homozygous *SMN* deletion, a different clinical diagnosis can be made in almost all cases.

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