

# Robust quantification of *the SMN* gene copy number by real-time TaqMan PCR

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**Abstract** Spinal muscular atrophy (SMA) is an autosomal recessive disease caused by mutation or deletion of the *survival motor neuron gene 1* (*SMN1*). The highly homologous gene, *SMN2*, is present in all patients, but it cannot compensate for loss of *SMN1*. *SMN2* differs from *SMN1* by a few nucleotide changes, but a C→T transition in exon 7 leads to exon skipping. As a result, most transcripts from the *SMN2* gene lack exon 7. Although *SMN1* is the disease-determining gene, the number of *SMN2* copies appears to modulate SMA clinical phenotypes. Thus, determining the *SMN* copy number is important for clinical diagnosis and prognosis. We have developed a quantitative real-time TaqMan polymerase chain reaction assay for both the *SMN1* and *SMN2* genes, in which reliable copy number determination was possible on deoxyribonucleic acid samples obtained by two different isolation methods and from two different sources (human blood and skin fibroblasts). For *SMN1*, allele specificity was attained solely by addition of an allele-specific forward

primer and, for *SMN2*, by addition of a specific forward primer and a nonextending oligonucleotide (*SMN1* blocker) that reduced nonspecific amplification from *SMN1* to a negligible level. We validated the reliability of this real-time polymerase chain reaction approach and found that the coefficient of variation for all the gene copy number measurements was below 10%. Quantitative analysis of the *SMN* copy number in SMA fibroblasts by this approach showed deletion of *SMN1* and an inverse correlation between the *SMN2* copy number and severity of the disease.

**Keywords** Spinal muscular atrophy · Survival motor neuron gene · Gene copy number assay · Real-time PCR

## Introduction

Spinal muscular atrophy (SMA) is a neuromuscular disease that affects the anterior horn cells of the spinal cord. SMA has a prevalence of 1 in 10,000 and a carrier incidence of approximately 1 in 50 [1–4]. Because it is an autosomal recessive disorder, both parents of an SMA patient must be carriers. When both parents are carriers, the likelihood of a child inheriting the disorder is 25% or one in four. Clinically, SMA can be categorized into types I, II, or III based on age of onset and severity of the disease [5]. Type I, also called Werdnig–Hoffmann disease, is the most severe. Patients with this type of SMA cannot sit unsupported or lift their heads. Type II patients can sit, and patients with type III can stand alone and walk but sometimes lose the ability to walk later in childhood, adolescence, or even adulthood.

Despite the clinical heterogeneity, SMA is caused by mutation or deletion of the *survival motor neuron gene 1*

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(*SMN1*) [6]. *SMN2*, which is 99% identical to *SMN1*, is present in all patients but is unable to fully compensate for loss of *SMN1*. *SMN2* differs from *SMN1* by a few nucleotide changes, but a transition from C→T in exon 7 leads to exon skipping [7, 8]. As a result, most of the transcripts from *SMN2* lack exon 7, and the resultant truncated protein appears biochemically unstable and is degraded rapidly in vivo [8].

Although *SMN1* is the disease-determining gene, the *SMN2* copy number can modify the clinical phenotypes. Studies from patients and animal models indicate that the severity of the disease inversely correlates with the *SMN2* copy number [9–14]. For example, individuals with one or two copies of the *SMN2* gene typically will have the most severe types of SMA. Three or more copies of the *SMN2* gene typically will result in a mild form of the disease. An increase in the number of *SMN2* copies likely results in more full-length SMN protein produced and a less severe form of the disease. Compounds such as aclarubicin, sodium butyrate, and valproic acid are shown to increase the amount of full-length *SMN2* transcripts and are becoming promising therapeutic agents for SMA [15–18]. Thus, accurate determination of the *SMN* copy number is important for clinical diagnosis and prognosis.

Genetic diagnosis of SMA commonly involves polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis [19]. This diagnostic test, which determines the presence or absence of the *SMN1* gene, may underestimate the *SMN* copy number because of partial digestion and lack of quantitative assessment. Several quantitative real-time PCR approaches to determine the *SMN* gene copy number have been developed [12, 20–23]. We have tested several of these methods for quantification of *SMN1* and *SMN2* gene copy numbers, but we were unable to achieve allele-specific amplification. In addition, these real-time PCR assays have been validated only with deoxyribonucleic acid (DNA) samples isolated from blood or isolated by a single method. Thus, reliability of the gene copy number assay may be compromised when DNA samples from sources other than patient blood are analyzed or samples from different labs are analyzed because these samples may not have been isolated using the same methodology.

In this study, we report the development of a quantitative, reliable, and reproducible real-time TaqMan PCR assay for *SMN1* and *SMN2* gene copy number determination. We have validated the reliability of this real-time PCR approach in the accurate determination of *SMN1* and *SMN2* gene copy numbers. We found that reliable copy number determination was possible on DNA samples obtained by two different isolation methods and from two different sources (human blood and skin fibroblasts).

## Materials and methods

### Tissue culture

Skin biopsies from SMA patients and controls were obtained as part of a study approved by the Institutional Review Board of the Alfred I. duPont Hospital for Children. Human fibroblast cell lines were established from these biopsies according to standard protocols [24]. Cells were maintained in Dulbecco's Modified Eagle's Medium (Invitrogen, Chicago, IL) supplemented with 20% fetal bovine serum (MediaTech, Herndon, VA), penicillin, and streptomycin. Cells were expanded into a T150-cm<sup>2</sup> flask until they were confluent, detached with trypsin/ethylenediamine tetraacetic acid, and harvested for DNA isolation.

### DNA isolation

Genomic DNA from blood samples was obtained from the Molecular Diagnostic Laboratory at the Alfred I. duPont Hospital for Children. DNA samples from blood were isolated using the Puregene D-5500 DNA isolation kit (Gentra Systems, Minneapolis, MN). The DNA samples from fibroblasts of SMA patients and controls were isolated using the DNeasy Tissue kit (Qiagen, Los Angeles, CA). The DNA was quantified using an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). All samples had 260/230 ratios greater than 2.0 and 260/280 ratios greater than 1.85. Externally validated DNA standards for *SMN1* (one and two copies/genome) and *SMN2* (zero, two, and three copies/genome) were kindly provided by Dr. Brunhilde Wirth from the Institute of Genetics and Center for Molecular Medicine Genetics, Cologne, Germany. These standards were used to validate two in-house DNA samples (see below). The *SMN* copy number of DNA samples used for validation assays was determined primarily by PCR RFLP analyses.

### Quantitative real-time TaqMan PCR of *SMN1* and *SMN2* for gene copy number determination

The primers, probes, and nonextending oligonucleotides for *SMN* and *cystic fibrosis transmembrane regulator (CFTR)* gene amplifications were designed based on those of previous studies [20, 22, 25] with minor modifications (Table 1). The forward primers were designed to distinguish between *SMN1* and *SMN2* by ending on the nucleotide difference (C/T) between the two genes in exon 7. A mismatch (T→G) at the -3 position from the 3' end of the primers was also added to both *SMN1* and *SMN2* forward primers to achieve allele specificity. The nonextending oligonucleotides (blockers) overlap with part of the forward primer and probe sequence such that they block nonspecific

**Table 1** PCR primers, TaqMan probes, and nonextending oligonucleotides (blockers) for SMN1, SMN2, and CFTR

Component	Sequence	Number of bases
<b>Primers</b>		
SMN1-ex7F-3g	TTC CTT TAT TTT CCT TAC AGG GT <sub>g</sub> <sup>b</sup> TC <sup>a</sup>	26
SMN2-ex7F-3g	TTC CTT TAT TTT CCT TAC AGG GT <sub>g</sub> <sup>b</sup> TT <sup>a</sup>	26
SMN-ex7R	GCT GGC AGA CTT ACT CCT TAA TTT AA	26
CFTR-F	TAG GAA GTC ACC AAA GCA GTA CAG C	25
CFTR-R	AGC TAT TCT CAT CTG CAT TCC AAT G	25
<b>Probes</b>		
SMN probe	FAM-ACC AAA TCA AAA AGA AGG AAG GTG CTC ACA-MGBNFQ	30
CFTR probe	VIC-TAT GAC CCG GAT AAC AAG GAG GAA CGC TC-MGBNFQ	29
<b>Blockers</b>		
SMN1 blocker	ATT TTC CTT ACA GGG TTT CAG ACA AAA TCA AAA-PO <sub>4</sub>	33
SMN2 blocker	ATT TTC CTT ACA GGG TTT TAG ACA AAA TCA AAA-PO <sub>4</sub>	33

<sup>a</sup> The SMN forward primers distinguished between SMN1 and SMN2 by ending on the nucleotide difference (C/T) at position 6 in exon 7.

<sup>b</sup> A mismatch T→G was added at the -3 position from the 3' end of both SMN1 and SMN2 forward primers to achieve allele specificity.

annealing of the allele-specific primer to the opposite allele, thus increasing assay specificity. The *SMN* probe was labeled with fluorescein dye at the 5' end and contained a minor groove binder (MGB) and a nonfluorescent quencher (NFQ) at the 3' end. The *CFTR* probe was designed using the Primer Express Software (Applied Biosystems, Atlanta, GA) with VIC at the 5' end and MGB and NFQ at the 3' end. Oligonucleotides were purchased from Integrated DNA Technologies and probes from Applied Biosystems.

The PCR reactions were performed in a total volume of 15 µl, containing 25 ng of genomic DNA, 1× TaqMan Universal PCR master mix (Applied Biosystems), 300 nM of *SMN1* primers or 450 nM of *SMN2* or *CFTR* primers, 650 nM of *SMN1* nonextending oligonucleotide for the *SMN2* assay, and 250 nM of *SMN* or *CFTR* probe. During assay development, 500 nM of the *SMN2* blocker was added to the *SMN1* assay, but it was later excluded from the reaction because it was not necessary for allele specificity. The real-time PCR was performed on a 7900HT Sequence Detection System (Applied Biosystems) using a 384-well format, and amplification was achieved using the standard amplification protocol (Applied Biosystems) as follows: 50°C for 2 min, 95°C for 10 min, followed by 45 cycles of 95°C for 15 s, and 60°C for 1 min. To enable normalization of the input target DNA added to each well, the internal control *CFTR* gene was amplified simultaneously in a separate reaction well but under identical thermal cycling conditions. Each reaction was run in triplicate, and each sample was run at least six separate times.

Externally validated genomic DNA samples with known *SMN* copy numbers (for *SMN1*, one [ES<sub>1</sub>] and two [ES<sub>2</sub>] copies/genome; for *SMN2*, zero [ES<sub>3</sub>], two [ES<sub>4</sub>], and three [ES<sub>5</sub>] copies/genome) were obtained from Dr. Brunhilde Wirth's laboratory [12]. These standards were initially used to confirm the two in-house samples that we used as standards. One DNA sample (IS<sub>1</sub>) was isolated from type

III SMA fibroblasts and had one mutated *SMN1* and one *SMN2* copy, as determined by PCR RFLP [24] and pyrosequencing analyses (unpublished data). The second sample (IS<sub>2</sub>) was from a control blood and had two *SMN1* and two *SMN2* copies, as determined by PCR RFLP. The in-house and externally validated DNA standards were run on each plate and compared with one another by defining one DNA sample as calibrator and the other samples as unknown. This procedure allowed us to unambiguously validate the *SMN* gene copy number in both in-house as well as externally validated DNA standards.

#### Data analysis

*SMN1*, *SMN2*, and *CFTR* real-time PCR assays were optimized and validated to enable data analysis by the comparative  $C_T$  method [26]. For valid dd $C_T$  calculations, the amplification efficiencies of target (*SMN1* and *SMN2*) and reference (*CFTR*) genes must be very similar. We determined the efficiency of *SMN* and *CFTR* amplification by the  $C_T$  slope method.  $C_T$  values were measured over a fivefold range dilution of a control DNA sample that contains two copies of each *SMN1*, *SMN2*, and *CFTR* per genome, and standard curves were created by plotting the  $C_T$  values vs the log amount of DNA. The amplification efficiency was at least 99% (data not shown), as determined by the  $R^2$  value obtained from each standard curve.

Amplification data for copy number determination were analyzed using the Sequence Detection Software SDS 2.2 (Applied Biosystems) and running relative quantification (RQ) studies where *SMN1* and *SMN2* were identified as targets and *CFTR* as the endogenous control. *SMN* data were normalized to *CFTR* (which is always two copies/genome) and calibrated to both externally and in-house validated DNA standards. When a two-copy DNA standard was used as a calibrator, the theoretical RQ (ratio) for zero

copies was 0, for one copy was 0.5, for two copies was 1.0, and for three copies was 1.5. These values were multiplied by a factor of two to obtain the gene copy number.

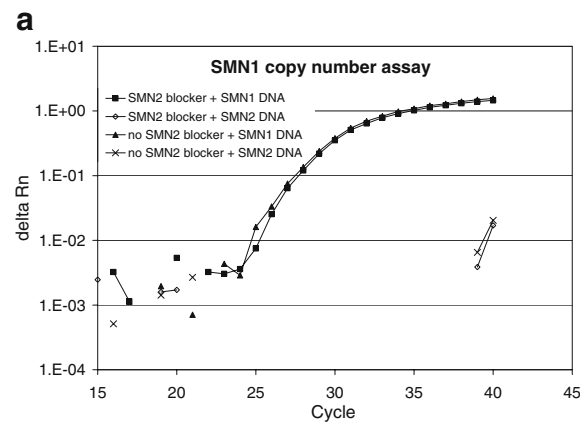
The reliability of the *SMN* gene copy number assay was measured by determining the coefficient of variation (CV). The CV for each sample was calculated by dividing the standard deviation (SD) of six repeated assays in a given subject by the average *SMN* copy number for that subject. The mean CV for each gene copy number was calculated by dividing the SD from the average copy number measured in all subjects with the same copy number by the average *SMN* copy number measured for that group. All the data sets of individuals with the same *SMN* number were combined, and the 99% confidence interval was determined. All acquired data were included in the RQ studies and the statistical analyses.

## Results

### Establishment of *SMN1* and *SMN2* real-time PCR for gene copy number quantification

We tested several existing real-time PCR methods for quantification of *SMN1* and *SMN2* gene copy numbers [12,

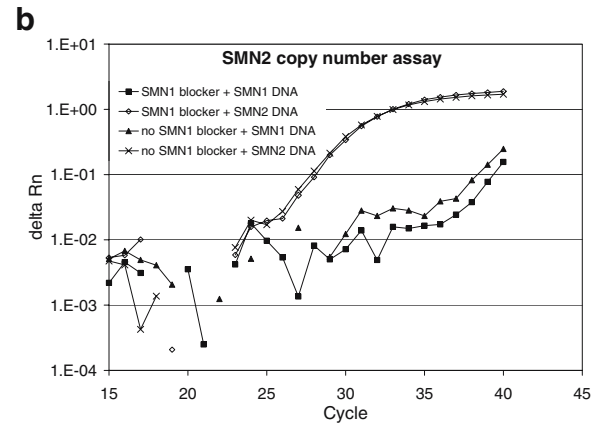
20–23] but were unable to consistently achieve allele-specific amplification of the genes (data not shown). To develop a more robust and quantitative assay for *SMN1* and *SMN2* gene copy number quantification, we modified forward primers previously reported that distinguished between *SMN1* and *SMN2* by ending on the nucleotide difference between the two genes at position 6 in exon 7 (see Table 1) [20]. This single nucleotide difference at the 3' end of the forward primer was not sufficient to achieve allele-specific amplification (data not shown). The addition of a mismatch (T→G) at the -3 position from the 3' end of both *SMN* forward primers was necessary to achieve allele specificity (Fig. 1). For the *SMN1* copy number assay, we determined a mean  $C_T$  value of  $26.72 \pm 0.04$  for a DNA sample with only *SMN1* copies. In contrast, the mean  $C_T$  value of a patient sample with only *SMN2* copies was  $41.10 \pm 0.41$ , indicating that addition of this -3 mismatch virtually eliminated nonspecific amplification of *SMN2* (Fig. 1a). The  $\Delta C_T$  between specific *SMN1* and nonspecific *SMN2* amplification was 14.38, indicating a discrimination of approximately 10,000-fold between the two genes. The presence of the *SMN2* blocker did not change the mean  $C_T$  significantly ( $\Delta C_T < 0.59$ ). These results indicated that the *SMN1* forward primer alone was sufficient for allele specificity and the *SMN2* blocker was not necessary to



The effect of *SMN2* blocker on the *SMN1* assay

Condition	Mean $C_T$ for <i>SMN1</i> DNA	Mean $C_T$ for <i>SMN2</i> DNA	$\Delta C_T$ between <i>SMN1</i> and <i>SMN2</i> DNA
- <i>SMN2</i> blocker	$26.72 \pm 0.04$	$41.10 \pm 0.41$	-14.38
+ <i>SMN2</i> blocker	$26.97 \pm 0.20$	$41.69 \pm 0.82$	-14.72
$\Delta C_T$ between blocker conditions	-0.25	-0.59	

**Fig. 1** Allele-specific real-time PCR amplification of *SMN1* and *SMN2* genes. **a** *SMN1* copy number assay. Allele specificity for the *SMN1* assay was tested using a DNA sample with only two copies of the *SMN1* gene (*SMN1* DNA) or only two copies of the *SMN2* gene (*SMN2* DNA) in the absence or presence of the *SMN2* blocker.  $\Delta R_n$  vs amplification cycle under different conditions was plotted, and the mean  $C_T$  values  $\pm$  standard deviation for all the tested conditions were



The effect of *SMN1* blocker on the *SMN2* assay

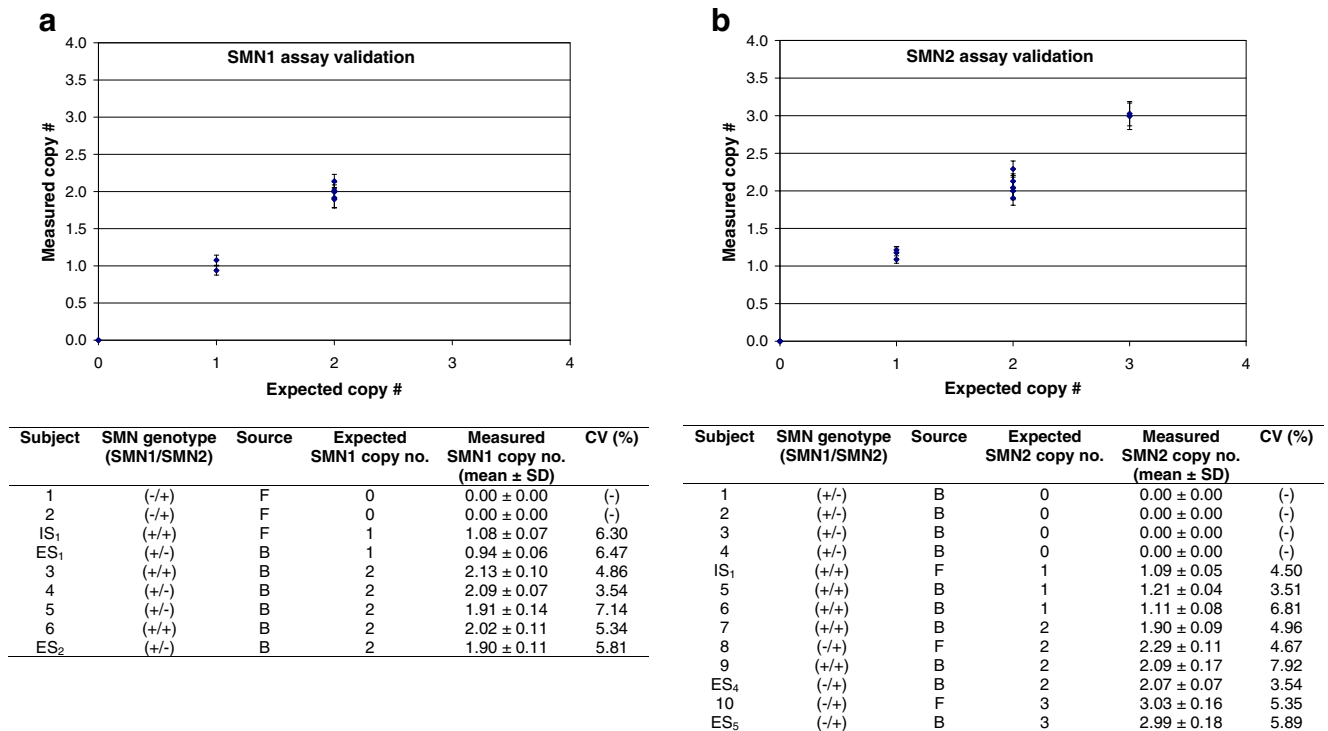
Condition	Mean $C_T$ for <i>SMN1</i> DNA	Mean $C_T$ for <i>SMN2</i> DNA	$\Delta C_T$ between <i>SMN1</i> and <i>SMN2</i> DNA
- <i>SMN1</i> blocker	$38.42 \pm 0.55$	$27.83 \pm 0.14$	-10.59
+ <i>SMN1</i> blocker	$40.00 \pm 1.17$	$28.02 \pm 0.10$	-11.98
$\Delta C_T$ between blocker conditions	-1.58	-0.19	

summarized in the table below. **b** *SMN2* copy number assay. Allele specificity for the *SMN2* assay was tested with the same DNA samples used for *SMN1* assay in the absence or presence of *SMN1* blocker.  $\Delta R_n$  vs amplification cycle under different conditions was plotted, and the mean  $C_T$  values  $\pm$  standard deviation for all the tested conditions were summarized in the table below as in **a**. Each sample was run as triplicate and repeated at least six different times

further increase specificity of the *SMN1* assay. For the *SMN2* assay, the mean  $C_T$  value for a DNA sample with only *SMN2* copies was  $27.83 \pm 0.14$ . However, the *SMN2* forward primer significantly reduced but did not completely abolish nonspecific amplification from a DNA sample with only *SMN1* copies, giving a mean  $C_T$  value of  $38.42 \pm 0.55$  (Fig. 1b). The  $\Delta C_T$  between specific and nonspecific *SMN* amplification was 10.59, indicating a discrimination of approximately 1,000-fold. The addition of the *SMN1* blocker increased the mean  $C_T$  value from  $38.42 \pm 0.55$  to  $40.00 \pm 1.17$  ( $\Delta C_T = 1.58$ ), a fivefold difference. This additional fivefold increase in discrimination between *SMN1* and *SMN2* genes rendered the assay suitable for *SMN2* gene copy number quantification. Presence of the *SMN1* blocker did not affect specific *SMN2* amplification ( $\Delta C_T = 0.19$ ). Thus, for the *SMN2* gene copy number assay, both specific *SMN2* forward primer and *SMN1* blocker were necessary to decrease nonspecific amplification of the *SMN1* gene to a negligible level.

Validation of *SMN1* and *SMN2* real-time PCR for gene copy number quantification

Once allele specificity was established for *SMN1* and *SMN2* assays, we validated their reliability in the accurate determination of the *SMN* gene copy number. We first used our *SMN1* assay to confirm externally validated DNA standards containing one ( $ES_1$ ) or two ( $ES_2$ ) copies of the *SMN1* gene. Likewise, the *SMN2* assay was confirmed with externally validated DNA standards containing zero ( $ES_3$ ), two ( $ES_4$ ), or three ( $ES_5$ ) copies of the *SMN2* genes (see “Materials and methods”). The results unambiguously validated the postulated gene copy number for all *SMN1* and *SMN2* DNA standards (data not shown). Next, we validated two in-house DNA samples ( $IS_1$  and  $IS_2$ ) with known *SMN1* and *SMN2* copy numbers (see “Materials and methods”) by comparing them with the external DNA standards (Fig. 2). The *SMN* copy number for each standard was confirmed regardless of the DNA standard used for



**Fig. 2** Validation of quantitative real-time TaqMan PCR assay for SMN copy number determination. **a** SMN1 assay validation. Genomic DNA samples with various SMN1 copy numbers were isolated from either fibroblasts (F) or blood (B). SMN genotypes for all samples have been previously determined by other methods (see “Materials and methods”). The SMN1 copy number measured by real-time PCR was plotted against the expected copy number. The mean-measured SMN1 copy number, standard deviation, and the coefficient of

variation (CV) were summarized in the table below. **b** SMN2 assay validation. Genomic DNA samples with various SMN2 copy numbers were isolated, and their SMN genotype and expected SMN2 copy numbers were determined as described in **a**. The SMN2 copy number measured by real-time PCR was plotted against the expected copy number. The mean-measured SMN2 copy number, standard deviation, and the coefficient of variation (CV) were summarized in the table below



**Table 2** Statistical evaluation of all SMN copy number measurements

Gene	Expected copy number	Measured copy number (Mean±SD)	Coefficient of variation (CV in %)
SMN1	0 (n=2)	0.00±0.00	(–)
	1 (n=2)	1.01±0.05	9.73
	2 (n=5)	2.01±0.05	5.17
SMN2	0 (n=4)	0.00±0.00	(–)
	1 (n=3)	1.16±0.03	6.08
	2 (n=4)	2.09±0.08	7.59
	3 (n=2)	3.01±0.01	0.81

calibration. The data presented in Fig. 2 and Tables 2 and 3 show the *SMN* copy number obtained relative to the in-house DNA standard IS<sub>2</sub>.

We then determined the reliability of both *SMN1* and *SMN2* assays in quantification of the *SMN* copy number by analyzing DNA samples with a known *SMN* copy number from six independent PCR assays performed on different days. Figure 2a shows that the *SMN1* gene copy number assay unambiguously identifies DNA samples with zero, one, and two copies of the *SMN1* gene without any overlap. The results also demonstrated that *SMN1* copy number determination was possible on DNA samples isolated from two different sources and by two different methods. For example, a DNA sample isolated from a patient fibroblast cell line (IS<sub>1</sub>), containing one copy of each *SMN1* and *SMN2* genes, was accurately identified as having one copy of *SMN1* (1.08±0.07) using the in-house standard (IS<sub>2</sub>) isolated from blood. The CV for all DNA samples analyzed was below 8%, indicating that the *SMN1* assay was reproducible in all DNA samples over time. Likewise, the *SMN2* gene copy number assay unambiguously identified DNA samples with zero, one, two, and three copies of *SMN2* gene without any overlap (Fig. 2b). The CV for all DNA samples analyzed was also below 8%, indicating that

the *SMN2* assay was also reproducible in all DNA samples over time. The reliability of all assays was assessed by the mean CV for each gene copy number measurement (Table 2). The CV for all assays was below 10%, indicating that our real-time PCR assay was reliable and accurately determined both *SMN1* and *SMN2* gene copy numbers. The 99% confidence interval for each validated *SMN1* copy number was 0.9–1.1 for one copy and 1.8–2.1 for two copies. For *SMN2*, the 99% confidence interval was 1.0–1.3 for one copy, 1.9–2.2 for two copies, and 2.8–3.2 for three copies.

#### Quantification of the SMN copy number in SMA fibroblasts

Previous studies indicated that skin fibroblasts derived from SMA patients expressed lower levels of SMN when compared with age-matched controls [24, 27]. Thus, SMA fibroblasts have been used as one of the model systems to study this disorder and to screen for therapeutic agents. We have collected five controls and 30 SMA fibroblasts with a range of clinical phenotypes (12 type I, 13 type II, and five type III) for SMN functional studies. To genetically characterize these SMA fibroblast cell lines, the real-time TaqMan PCR assays were used to determine the *SMN* copy number. We found that all control fibroblasts contained two copies of *SMN1*, while SMA fibroblasts had no copies of *SMN1* (Table 3). Moreover, despite the limited number of cell lines analyzed, the number of *SMN2* copies in SMA fibroblasts correlated inversely with clinical phenotypes (Table 3). For example, 91.7% of type I SMA fibroblasts contained two copies of *SMN2*, 76.9% of type II carried three copies, and 80% of type III carried three or more copies. These results are consistent with findings obtained from patient blood samples [9–13] and further confirm the reliability of this assay in the quantification of the *SMN* genes.

**Table 3** Distribution of SMN1 and SMN2 copies in control and SMA fibroblasts

Patient status	Copy number distribution					Total
	0	1	2	3	3+	
<b>SMN1</b>						
Control	0 (0.0%)	0 (0.0%)	5 (100.0%)	0 (0.0%)	0 (0.0%)	5
Type I	12 (100.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	12
Type II	13 (100.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	13
Type III	5 (100.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	5
Total						35
<b>SMN2</b>						
Control	0 (0.0%)	1 (20.0%)	3 (60.0%)	1 (20.0%)	0 (0.0%)	5
Type I	0 (0.0%)	0 (0.0%)	11 (91.7%)	1 (8.3%)	0 (0.0%)	12
Type II	0 (0.0%)	0 (0.0%)	1 (7.7%)	10 (76.9%)	2 (15.4%)	13
Type III	0 (0.0%)	0 (0.0%)	1 (20.0%)	1 (20.0%)	3 (60.0%)	5
Total						35

## Discussion

The most common approach for genetic diagnosis of SMA involves PCR amplification followed by RFLP analysis [19]. The *SMN1* gene, which is present in normal individuals, is either deleted or undergoes gene conversion in approximately 95% of patients with SMA. In the remaining SMA patients, one copy of *SMN1* is deleted, while the other commonly carries a point mutation [9, 28–30]. Thus, this diagnostic test, which determines the presence or absence of the *SMN1* gene, may underestimate the *SMN* copy number because of partial digestion and the lack of quantitative assessment. Quantitative *SMN* gene dosage analyses using competitive PCR have been described [31–34]. These approaches are extremely complicated, as they involve the use of radioactivity, the construction of plasmid standards, and restriction enzyme digestion. Thus, they are not suitable for routine use as a molecular diagnostic test in laboratories. To date, several quantitative real-time PCR approaches using LightCycler or TaqMan technology have been developed for *SMN* gene copy number determination [12, 20–23]. The advantages of a real-time PCR method for *SMN* copy number determination are obvious; it is nonradioactive, highly sensitive, and suitable for high-throughput analysis.

We tested several existing real-time PCR methods for the quantification of the *SMN* gene copy number but were unable to achieve allele-specific amplification. Thus, we developed a real-time TaqMan PCR assay to provide robust and consistent allele-specific amplification of the *SMN1* and *SMN2* genes. For the *SMN1* assay, allele specificity was attained by addition of a specific forward primer with a -3 mismatch. For the *SMN2* assay, the addition of a specific forward primer with a -3 mismatch and an *SMN1* blocker was required to reduce nonspecific amplification of *SMN1* to a negligible level. We noticed that the amplification efficiency for the *SMN2* allele was lower than that for *SMN1*, which may result from the previously reported PCR bias [35]. Thus, to achieve the same amplification efficiency for both *SMN* genes, we used a higher concentration of *SMN2* primers in the reaction. Higher primer concentration may lead to lower PCR stringency. This may explain why the addition of an *SMN1* blocker was required in the *SMN2* assay to further improve specificity. Moreover, an external reference locus (CFTR) with a fixed copy number was used to normalize against unavoidable deviations in DNA concentration. Robustness of our assay was also increased by the use of a two-copy standard as a calibrator sample in every single run. This allowed further normalization and the ability to compare data acquired from different runs without having to prepare standard curves. We validated the reliability of this real-time PCR method and found that the CV for all *SMN* gene copy number measurements was

below 10%. No overlap between the measured copy numbers was observed for either gene, which allowed a clear differentiation between different *SMN1* and *SMN2* copy numbers. In contrast, other PCR approaches developed to date do not allow differentiation between carriers and normal individuals [9, 36]. Moreover, in our real-time PCR assay, reliable copy number determination was possible on DNA samples obtained by different isolation methods and from different sources (blood and fibroblasts). All the published real-time PCR assays have been validated only with DNA samples isolated from blood and with DNA samples isolated by a single method [12, 20–23]. Thus, reliability of the *SMN* gene copy number assay may be compromised when DNA samples from different laboratories are tested, as these samples may not have been isolated by the same method or come from the same source.

The determination of the *SMN1* copy number during carrier testing is important for diagnostic purposes and genetic counseling [4, 9, 36]. *SMN* copy number determination in SMA and control fibroblasts confirmed that all patients were deleted for *SMN1*, while all controls had two copies. Accurate determination of the *SMN2* copy number is important as well because severity of the disease seems to inversely correlate with the *SMN2* copy number [9–13]. Our data showed that patients with more *SMN2* copies had a less severe clinical phenotype, consistent with what has been observed from patient blood samples [9–13]. An increase in the number of *SMN2* copies can increase the amount of full-length SMN protein produced, resulting in a less severe form of SMA and, thus, a better prognosis. The correct measurement of the *SMN2* copy number can also be important for patient selection for drug clinical trials. It has been shown that compounds such as aclarubicin, sodium butyrate, and valproic acid increase the amount of full-length *SMN2* transcript [15–18]. Thus, the accurate determination of the *SMN2* copy number on SMA patients participating in current and future drug trials could be a very valuable tool for prognosis.

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