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Preclinical validation of a multiplex real-time assay to quantify SMN mRNA in patients with SMA

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Abstract

Objective—To determine whether survival motor neuron (SMN) expression was stable over time.

Methods—We developed a multiplex real-time reverse transcriptase (RT)-PCR assay to quantify SMN transcripts in preclinical blood samples from 42 patients with spinal muscular atrophy (SMA) drawn for three time points per patient; most blood samples were shipped to a centralized laboratory.

Results—We obtained a sufficient amount ($9.7 \pm 5.6 \mu\text{g}$) of good-quality total RNA, and RNAs were stable for up to a 3-year interval. This allowed RNA samples collected during a 9- to 12-month period to be analyzed in a single run, thus minimizing interexperimental variability. SMN expression was stable over time; intersample variability for baseline measures, collected during a 17-month interval, was less than 15% for 38 of 42 SMA patients analyzed. This variability was well below the 1.95-fold increase in full-length SMN (fSMN) transcripts detected in SMA fibroblasts treated with 10 mM valproic acid.

Conclusion—Real-time quantification of SMN messenger RNA expression may be a biomarker that is amenable to multicenter SMA clinical trials.

Spinal muscular atrophy (SMA) is an autosomal recessive disorder associated with degeneration of alpha motor neurons in the spinal cord.¹ There are two survival motor neuron (*SMN*) genes in humans,² the major functional difference between *SMN1* and *SMN2* being a C-to-T transition in exon 7³ that promotes skipping of exon 7 ($\Delta 7\text{SMN}$) in *SMN2* gene transcripts.⁴ Mutations in the *SMN1* gene are responsible for all forms of childhood-onset SMA.⁵ Although *SMN2* cannot completely compensate *SMN1* deficiency, disease severity does correlate with *SMN2* gene copy number.^{6,7}

Muscle atrophy precedes loss of motor neurons in two distinct SMA animal models,^{8,9} and disease pathology is rescued by the *SMN2* gene in a dose-dependent manner.⁸ Two U.S. Food and Drug Administration–approved drugs used to treat children with urea cycle disorders and epilepsy (namely, phenylbutyrate and valproic acid [VPA]) affect SMN

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expression in SMA fibroblast cultures^{10,11} and patients with SMA.¹² In SMA fibroblast cultures, VPA treatment resulted in a 1.8-fold increase in full-length SMN (fSMN) messenger RNA (mRNA), with a coincident increase in SMN protein.^{10,13} Both compounds are thought to exert their effect by enhancing SMN expression through inhibition of histone deacetylation and by altering SMN splicing.^{10,14}

Anticipating SMA clinical trials, we validated a multiplex real-time reverse transcriptase (RT)-PCR assay to quantify SMN transcripts in preclinical whole-blood samples from SMA patients. Our data indicate that this assay can be used as a secondary measure to follow the effects of drug treatment on SMN expression.

Methods

Subjects and blood draws

Three baseline peripheral venous blood draws, using PAXgene blood RNA tubes (BD Biosciences), were obtained from 42 SMA patients. Patients had confirmed homozygous mutations in the *SMN1* gene and were classified as type 1 ($n = 4$), type 2 ($n = 31$), or type 3 ($n = 7$) SMA. Of these, 37 patients were recruited at the Utah site, and vacutainer tubes were shipped overnight, sandwiched between two frozen icepacks. *SMN2* gene copy number was determined as previously described.¹⁵ Informed consent (and informed assent in children older than 12 years of age) was obtained from each subject or their parents. This protocol was approved independently by the institutional review boards and general clinical research center review boards at the University of Utah School of Medicine, Primary Child Medical Centre, and Sainte-Justine Hospital Research Centre.

RNA extraction and complementary DNA synthesis

All samples were stored at 4°C for no more than 5 days before extracting RNA using the PAXgene blood RNA kit (QIAGEN) and specifications provided by the manufacturer. RNA quality and quantity were determined by absorbance at 260 and 280 nm. Complementary DNA (cDNA) synthesis was performed using the RT Omniscript kit (QIAGEN). Each reaction contained 1 µg of total RNA, which had been previously incubated at 65°C for 5 minutes; 1X RT Omniscript reaction buffer; 0.5 mM each of dNTP, 1 µM Oligo-dT primer, and 10 U RNase inhibitor; and 4 U of RT Omniscript enzyme; total volume was 20 µL. Reactions were incubated for 60 minutes at 37°C, and the RT Omniscript enzyme was inactivated by heating to 93°C for 5 minutes.

Real-time RT-PCR

The assay was carried out on an ABI Prism 7000 Sequence Detection System (Applied Biosystems) using 5'-labeled FAM or VIC fluorescent probes containing a 3' minor groove binder/nonfluorescent quencher (MGBNFQ) label. Primers for exon 7 (5'-AAAAGAAGGAAGGTGCTCACATTC-3') and exon 8 (5'-TGGTGTCATTTAGTGCTGCTCTATG-3') were used to amplify SMN transcripts, and fSMN was detected with an exon 7–exon 8 junction probe (5'-FAM-CAGCATTTCTCCTTAATTT-MGBNFQ-3'). Primers for exon 6 (5'-CATGGTACATGAGTGGCTATCATACTG-3') and exon 8 (5'-AGTGGTGTCATTTAGTGCTGCTCTAT-3') were used to amplify SMN transcripts, and Δ 7SMN was detected with an exon 6–exon 8 junction probe (5'-FAM-CCAGCATTTCCATATAATAGMGBNFQ-3'). Human phosphoglycerate kinase (PGK1) transcripts were also quantified using the TaqMan endogenous control kit (Applied Biosystems) to normalize each reaction for the amount of input template and efficacy of RT-PCR. PCR reactions were carried out in a final volume of 25 µL and contained 0.4 µM oligonucleotide primers, 0.2 µM probes, 2.5 µL of a 1/80 dilution of the cDNA reaction, and

1X Taqman Universal Master Mix (Applied Biosystems). For multiplex reactions, 1X TaqMan endogenous control for human *PGK1* was added directly into the individual SMN reactions. Multiplex reactions were compared against simplex reactions quantifying SMN and PGK transcripts separately. Cycling conditions were as follows: a 95°C, 10-minute initial denaturation step, followed by 45 cycles of 95°C for 1 minute, and then 60°C for 1 minute.

Quantification and analysis of real-time data

The amount of PCR product was calculated on the basis of the threshold cycle (C_T), namely, the cycle where fluorescence was detected above baseline. Data were analyzed using Real-time PCR System Sequence Detection Software v1.0, supplied by Applied Biosystems. Relative quantification of fSMN, $\Delta 7$ SMN, and PGK1 transcripts were determined by the standard curve method using serial dilutions (1/2, 1/6, 1/20, 1/60, 1/200, and 1/1000) of freshly prepared cDNA from normal control RNA, which was extracted from PAX-gene RNA tubes. The 1/1000 dilution was excluded in $\Delta 7$ SMN reactions because this transcript was undetectable in control RNA at this concentration. Real-time reactions were carried out in triplicate for each dilution, and standard curves were prepared by plotting the mean C_T values against the log concentration of the input starting material. Amplification efficiencies were assessed by comparing the C_T values for each concentration used to construct standard curves. Standard curves were then used to quantify the amount of respective transcripts in patient samples.

Simplex reactions were carried out in triplicate, and the mean relative amount of SMN transcript was normalized by dividing this value by the mean relative amount of PGK transcript. Results are presented as the mean of two separate triplicate reactions for each cDNA sample. For multiplex reactions, the amount of SMN transcript was normalized for each well, and results are presented as the mean of normalized values for six reactions per patient sample. All time points for a given patient were run on the same 96-well plate. Baseline measures for each patient are presented as means $\pm$ SEM between time points.

For comparison, quantification of multiplex reactions was determined using a calibrator with the value for a 1/80 dilution of the control cDNA sample. The relative amount of SMN transcript was calculated using the $2^{-\Delta\Delta C_T}$ method,¹⁶ where ΔC_T corresponds to the C_T for SMN minus the C_T for PGK1, and $\Delta\Delta C_T$ corresponds to the ΔC_T of patient sample minus the ΔC_T of the calibrator sample. This value was calculated for each well, and results are presented as the mean $2^{-\Delta\Delta C_T}$ of six reactions per patient sample.

VPA treatment of SMA fibroblast cells

Fibroblast cell line 3813 (Coriell Cell Repository) was established from an *SMN1*^{-/-}, *NAIP*^{-/-}, and *SMN2*^{+/+} SMA type 1 patient. A total of 2×10^5 cells were plated onto P100 culture dishes, and after 3 hours they were treated with vehicle, 1 mM or 10 mM VPA, for 72 hours as previously described.¹⁰ RNA was prepared with Trizol reagent (Invitrogen) as specified. Multiplex SMN real time was conducted as described above, in addition to reactions that incorporated 1X TaqMan endogenous control for either peptidylprolyl isomerase A (PPIA) or β -glucuronidase (GUSB). Results are reported as means for two separate drug treatments, each run in duplicate.

Results

SMA patient RNA from whole-blood samples

Cohort sizes sufficiently large to obtain meaningful results during SMA clinical trials can only be achieved in the context of a multicenter study. A potential secondary outcome

measure is the quantification of fISMN mRNA transcripts in whole blood, a convenient and accessible source of biological material, given that drugs currently being considered for SMA clinical trials specifically target SMN expression and splicing. The first objective of this study was to determine whether we could recover good-quality RNA from SMA patient blood samples collected in PAX-gene vacutainer tubes, shipped from a clinical site to a molecular laboratory, in sufficient quantities to conduct real-time RT-PCR assays. A total of 37 SMA patients were followed at the Utah site, and five were seen in Montreal, the site of the molecular laboratory. The majority of patients ($n = 31$) had SMA type 2, whereas four patients had SMA type 1, and seven had SMA type 3.

PAXgene vacutainer tubes reportedly stabilize RNA for 5 days at room temperature; however, our preliminary data (not shown) indicated that RNA yield was augmented by maintaining samples at 4°C. Therefore, all samples were stored at 4°C for no more than 5 days, and samples from the Utah site were shipped overnight, sandwiched between two frozen icepacks. The SMA natural history trial required blood draws for multiple tests; therefore, drawing blood directly into vacutainer tubes, as recommended, would have been inappropriate. Consequently, blood was collected into a syringe via a butterfly and then distributed into appropriate collection tubes. The average RNA yield for 126 blood samples was $9.7 \pm 5.6 \mu\text{g}$ RNA for 2.5 mL of whole blood, with yields ranging from 1.1 to 41.3 μg RNA. These values were fairly comparable between sites: $10.2 \pm 5.8 \mu\text{g}$ for shipped samples ($n = 111$) vs $6.6 \pm 3.1 \mu\text{g}$ for onsite samples ($n = 15$). Thus, neither blood collection via syringes nor overnight shipment of samples interfered with the RNA yield.

Multiplex real-time RT-PCR quantification of SMN transcripts

Because SMA clinical trials last a minimum of 6 to 12 months and may extend beyond 1 year, the second objective of this study was to determine whether SMN expression was stable over time and whether between-sample variability was low enough to permit detection of a drug effect on SMN expression. The relative quantity of fISMN mRNA in patient blood was determined by multiplex real-time RT-PCR, as described in the Methods section, and intrasubject variability was assessed for baseline samples collected during a 1.5- to 17-month interval (mean, 7.2 ± 2.8 months). A total of six measures were obtained for each sample, and the relative amount of normalized fISMN for two to three baseline time points was determined using the standard curve method described in the Methods section. Comparison of the ΔC_T values for each dilution in the standard curves, the slopes and ΔR_n values, were indicative of efficient fISMN and PGK1 amplifications, and data were comparable between runs. Transcripts were not detected when RT was excluded from the reaction.

The mean normalized relative quantity of fISMN transcripts in 42 SMA patients, as determined by the standard curve method, is presented in figure E-1A (available on the *Neurology* Web site at www.neurology.org). Each cylinder in the graph corresponds to the mean and SEM of six measures for three baseline time points for 37 SMA patients (gray cylinders) and two baseline time points for five SMA patients (white cylinders). Overall, we could not quantify SMN transcripts for four samples because of poor RNA quality/quantity, corresponding to a failure rate of 3.2%. The relative quantity of fISMN transcripts on the basis of multiplex or simplex reactions are provided in table E-1; as can be seen, this relative quantity varied greatly between SMA patients, ranging from 0.30 ± 0.14 to 1.22 ± 0.08 . Intersample variability per patient is reflected by the error bars (SEM) and was $\leq 15\%$ for 38 SMA patients and between 19% and 25% for four patients. Thus, within-patient variability was under 25%, well below reported drug effects observed in treated SMA patient fibroblast cell lines.^{10,13,14}

Comparison of multiplex and simplex reactions

We initially quantified fISMN using separate reactions for fISMN and PGK1 transcripts (simplex reactions) for 39 SMA patients, and these data were used to validate the multiplex assay. The objective of establishing a multiplex reaction was to decrease variability between samples, because each reaction is normalized with a specific endogenous control. The mean relative fISMN expression over time was concordant with SEMs of simplex and multiplex results below 21% for all 39 SMA patients (table E-1). The validated multiplex reaction reduces SMN quantification costs by half.

Quantification of fISMN expression using a calibrator

Quantification using the standard curve method necessitates including five to six serial dilutions of a control cDNA, each analyzed in triplicate. When PCR efficiencies approach 100%, an alternate strategy is to compare test samples with a calibrator sample. We reanalyzed multiplex data using values corresponding to a 1/80 dilution of a control cDNA as calibrator (figure E-1B). Not unexpectedly, these results are identical to those obtained using the standard curve method. Consequently, once PCR efficiencies have been validated, quantification can be achieved using the calibrator approach. This strategy reduces cost as well as the amount of input RNA required for RT reactions, because the use of 1 µg of RNA was dictated by the need to produce sufficient cDNA from the control sample to establish the standard curves. In reality, real-time SMN RT-PCR can be done effectively using a 1/40 dilution of an RT reaction with as little as 0.25 µg of input RNA (data not shown). In the context of clinical drug trials, the non-treated baseline measures will serve as a calibrator for treatment measures.

Effect of SMN2 gene copy number on fISMN expression

Because the amount of fISMN transcripts varied greatly between SMA patients, we assessed whether this was associated with disease severity and *SMN2* gene copy number as one might expect, increasing relative amounts of fISMN transcripts with increasing copies of the *SMN2* gene, which is also associated with disease severity. These data were available for 38 SMA patients: four type 1 and one type 2 SMA patients had two *SMN2* genes, 19 type 2 and four type 3 SMA patients had three *SMN2* genes, seven type 2 and two type 3 SMA patients had four *SMN2* genes, and one type 3 patient had five *SMN2* genes. This distribution accurately reflects previously published results.⁶ As can be seen in figure 1, there was no correlation between the amount of fISMN transcripts in SMA patient blood samples and *SMN2* gene copy number. Similar results were observed for SMA type (data not shown). Indeed, 19 type 2 SMA patients had three *SMN2* genes, and the amount of fISMN transcripts in blood samples ranged from 0.36 to 1.22, values that clearly overlap those obtained for patients with two (0.30 to 1.08), four (0.48 to 1.09), or five (0.82) *SMN2* genes.

VPA treatment of SMA fibroblast cells

The final objective of this study was to determine whether our multiplex real-time RT-PCR assay could detect previously reported changes in SMN expression in SMA patient fibroblasts treated with VPA. Because VPA may affect the expression of up to 10% of all genes,¹⁷ we analyzed three different endogenous controls: PGK1, PPIA, and GUSB. Fibroblast cell line 3813 (Coriell Cell Repository), derived from a type 1 SMA patient harboring homozygous deletions of the *SMN1* and *NAIP* genes, was exposed to vehicle, 0.1 mM VPA or 10 mM VPA, for 72 hours, and fISMN transcripts were quantified in conjunction with either PGK1, PPIA, or GUSB transcripts. Results are summarized in figure 2A. As can be seen, we detected a 1.95-fold increase in the relative quantity of fISMN transcripts in response to 10 mM VPA, consistent with previously published data.¹⁰ More importantly, this increase was only observed when PGK1 was used as an endogenous

control, suggesting that both PIPA and GUSB gene expression were affected by VPA treatment.

The effect of VPA treatment may be to enhance *SMN2* gene expression, to favor inclusion of exon 7 in transcripts generated by the *SMN2* gene, or both. These samples were analyzed by multiplex real-time RT-PCR to quantify $\Delta 7$ SMN transcripts, as described in the Methods section, to distinguish between these possibilities. Fibroblasts exposed to 10 mM VPA displayed a modest (1.2-fold) increase in the relative amount of $\Delta 7$ SMN transcripts (using PGK1 as endogenous control (figure 2B), suggesting that the primary VPA effect is to augment *SMN2* gene expression in patient fibroblasts. Consistent with fLSMN data, we did not detect a VPA effect when PPIA or GUSB were used as the internal standard.

Ratio of fLSMN to $\Delta 7$ SMN transcripts in SMA patients and carrier parents

The relative amounts of fLSMN transcripts detected in some SMA patient blood samples were well within the ranges observed for carrier and noncarrier individuals (data not shown). Although quantification of the relative amount of fLSMN provides sufficient data to test effects of drug treatment, it does not distinguish between unaffected and affected individuals. For this, one must determine the ratio of fLSMN to $\Delta 7$ SMN transcripts, as summarized in the table. The relative f/ $\Delta 7$ SMN ratios in SMA patients ranged between 0.24 and 0.47 and were about half those observed for carrier parents, whose values ranged from 0.57 to 1.26. The ratio of relative amounts of f/ $\Delta 7$ SMN transcripts in a noncarrier individual was twice that observed for carriers.

Discussion

Drugs considered for the treatment of SMA exert their effect by increasing the amount of fLSMN produced by the *SMN2* gene; thus, quantification of these transcripts provides a possible biomarker and secondary outcome measure in SMA clinical trials. To this end, we developed and validated a real-time RT-PCR multiplex assay to quantify the relative amount of fLSMN and $\Delta 7$ SMN transcripts.

Our data indicate that shipment of PAXgene blood RNA vacutainer tubes to a central laboratory does not jeopardize RNA yield or quality; RNA remained stable during a 1- to 3-year period. We could not quantify SMN transcripts for four blood samples because of poor RNA quality/quantity, corresponding to an overall failure rate of 3.2%, which is within an acceptable range for clinical trials involving children. The most important finding was that SMN expression remained stable over time; intersample variability for baseline measures, some of which spanned a 17-month interval, was less than 25% overall, well below the 1.95-fold increase in fLSMN expression detected in VPA-treated SMA fibroblasts. Thus, if drug treatment affects SMN expression in blood, this assay is sufficiently sensitive to detect this change.

The *SMN2* gene is the only known modifier of the SMA phenotype; most type 1 patients possess one or two *SMN2* genes, whereas most type 3 individuals have three or four copies.⁶ Variability in the relative amount of SMN transcripts among SMA patients was not unexpected; however, this variability was not associated with *SMN2* copy number. This contrasts recent data suggesting an association between *SMN2* copy number and SMN expression.¹⁸ Discordance between the studies may be attributable to the different distribution of SMA types; our study had three times as many type 2 patients (31 vs 9) and half the number of type 3 individuals (7 vs 14). Although analysis of a much larger sample may resolve this issue, the absence of a strict transcript–copy number correlation is more consistent with the continuum of clinical severity observed even within SMA types.¹⁹ Our findings suggest that not all *SMN2* genes are equal and that, as previously proposed,²⁰ some

genes may not be intact or may harbor mutations affecting expression or splicing. Current techniques quantify *SMN* genes using the diagnostic T (*SMN2*) or C (*SMN1*) nucleotide in exon 7 and do not probe the entire gene.¹⁵ Pulse-field data have demonstrated that the genomic organization of the *SMN* locus is highly variable, even in the relatively isolated Finnish population.²¹ Consequently, the actual genomic organization of a patient's *SMN2* genes may affect *SMN2* expression, and drug responses may also vary between patients, such that some SMA individuals may not even respond to HDAC inhibitors. Finally, what distinguishes SMA patients from non-SMA individuals is the ratio of flSMN to $\Delta 7$ SMN. This ratio seems to be critical, because $\Delta 7$ SMN is incorporated into cytoplasmic and nuclear SMN complexes when present in nearly equimolar amounts,²⁰ whereas $\Delta 7$ SMN is exclusively retained within the nucleus when overexpressed.²² Clearly, an SMN protein assay amenable to multicenter trials could provide additional insight into the relationship between disease severity and drug response. Our preliminary data using blood cell lysates and Western blot analyses seem to show a better relationship between SMA type and protein levels, at least with regard to distinguishing type 1 patients from other SMA types and controls (data not shown), which is consistent with recent cell immunoassay data.¹⁸

Epigenetic regulation of gene expression is mediated, in part, by reversible modification of histone proteins that define a specific "histone code."²³ Acetylation occurs via a family of histone acetyltransferases and can be reversed by histone deacetylases (HDACs). Because in vitro and in vivo treatment with HDAC inhibitors can affect up to 25% of genes,²³⁻²⁶ several endogenous controls should be used to normalize RT-PCR results, and these must be validated for each drug and cell type employed. In this study, GUSB and PPIA gene expression seemed to be affected by VPA treatment, masking the effect of VPA on *SMN* gene expression in SMA type 1 fibroblast cells observed when using PGK1 (this study) or GAPDH^{10,13} as endogenous controls. Although one might argue that PGK1 was also affected by VPA treatment, we observed a 1.95-fold increase in the relative amount of flSMN transcripts compared with the 1.2-fold increase in $\Delta 7$ SMN, providing evidence for arguing against this possibility. Indeed, had PGK1 expression also responded to VPA treatment, flSMN and $\Delta 7$ SMN would have been affected equally. Furthermore, these results replicate previous reports suggesting that VPA affects both SMN expression and splicing.^{10,13} Because the mode of action of HDAC inhibitors is complex, one should not use flSMN: $\Delta 7$ SMN ratios to assess drug response; if enhanced expression and splicing are equally affected, the amount of both flSMN and $\Delta 7$ SMN transcripts could increase without affecting the ratio of flSMN: $\Delta 7$ SMN in treated compared with baseline samples.

Changes detected in blood can reflect changes observed in target tissues, suggesting that real-time data from peripheral blood can be used as a biomarker of disease progression or drug response. Gene-expression profiling of blood from subjects with Huntington disease revealed 12 biomarkers whose expression correlated with disease progression,²⁶ whereas distinct profiles were observed between VPA responders and nonresponders in children with epilepsy.¹⁷ In this study, we validated a real-time assay to quantify SMN transcripts in whole-blood samples that is sensitive enough to detect changes of >1.5-fold in SMN expression. However, because HDAC inhibitors affect expression of a large number of genes, several endogenous controls should be analyzed to ensure that their expression is not affected by the test drug. Ongoing expression-profiling studies should aid in the choice of appropriate normalizing controls.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Relationship between fLSMN transcript quantity and SMN2 copy number. Quantification of fLSMN was carried out by multiplex RT-PCR using PGK1 as an endogenous control. SMN2 copy number was evaluated as previously described.¹⁵

Figure 2. Effect of VPA treatment of type I SMA fibroblast cells. SMA type I fibroblast cell line 3831 was treated with 0, 1, or 10 mM VPA (medium, light, and dark gray cylinders, respectively), and the amount of fSMN (A) or $\Delta 7$ SMN (B) transcripts was determined using either PGK1, GUSB, or PPIA as endogenous controls. Data are reported as means $\pm$ SEM of two experiments.

Table*Relative amount of flSMN and $\Delta 7$ SMN transcripts in SMA patients and carriers*

ID	SMA type	Relative flSMN	Relative $\Delta 7$ SMN	flSMN/ $\Delta 7$ SMN
45341	1	0.38 $\pm$ 0.02	1.09 $\pm$ 0.03	0.35
50570	1	0.89 $\pm$ 0.03	2.00 $\pm$ 0.31	0.44
50807	1	1.07 $\pm$ 0.10	2.17 $\pm$ 0.06	0.49
51040	1	0.86 $\pm$ 0.01	2.18 $\pm$ 0.04	0.39
47915	2	1.21 $\pm$ 0.01	2.97 $\pm$ 0.21	0.41
47969	2	1.00 $\pm$ 0.04	2.49 $\pm$ 0.27	0.40
49525	2	0.75 $\pm$ 0.06	2.09 $\pm$ 0.01	0.36
49833	2	0.67 $\pm$ 0.01	1.18 $\pm$ 0.18	0.57
50317	2	0.67 $\pm$ 0.04	1.84 $\pm$ 0.07	0.36
51452	2	0.91 $\pm$ 0.04	2.69 $\pm$ 0.09	0.34
51899	2	0.92 $\pm$ 0.06	2.30 $\pm$ 0.19	0.40
53635	2	0.64 $\pm$ 0.02	2.11 $\pm$ 0.04	0.30
58488	2	0.68 $\pm$ 0.04	2.46 $\pm$ 0.02	0.28
57090	2	0.46 $\pm$ 0.01	1.38 $\pm$ 0.09	0.33
58444	2	0.65 $\pm$ 0.002	1.55 $\pm$ 0.07	0.42
58104	2	0.41 $\pm$ 0.04	1.74 $\pm$ 0.11	0.24
58269	2	0.28 $\pm$ 0.001	0.61 $\pm$ 0.01	0.46
49832	3	0.46 $\pm$ 0.03	1.27 $\pm$ 0.02	0.36
58423	3	0.39 $\pm$ 0.01	1.16 $\pm$ 0.06	0.34
58676	3	0.89 $\pm$ 0.03	2.70 $\pm$ 0.001	0.33
41307	3	0.49 $\pm$ 0.04	1.19 $\pm$ 0.04	0.41
44442	3	0.69 $\pm$ 0.001	2.20 $\pm$ 0.15	0.31
46837	Carrier	0.54 $\pm$ 0.01	0.60 $\pm$ 0.01	0.90
47012	Carrier	1.12 $\pm$ 0.03	1.84 $\pm$ 0.13	0.61
47968	Carrier	0.88 $\pm$ 0.02	1.00 $\pm$ 0.02	0.88
50316	Carrier	0.94 $\pm$ 0.02	1.08 $\pm$ 0.09	0.87
50486	Carrier	0.41 $\pm$ 0.02	0.69 $\pm$ 0.03	0.59
50569	Carrier	0.49 $\pm$ 0.02	0.37 $\pm$ 0.03	1.32
51556	Carrier	0.53 $\pm$ 0.01	0.76 $\pm$ 0.05	0.70
51722	Carrier	0.83 $\pm$ 0.04	1.44 $\pm$ 0.04	0.58
51946	Carrier	0.38 $\pm$ 0.02	0.37 $\pm$ 0.02	1.03
44440	Carrier	0.72 $\pm$ 0.05	1.20 $\pm$ 0.03	0.60
49677	Carrier	0.55 $\pm$ 0.01	0.94 $\pm$ 0.06	0.58
53636	Carrier	0.70 $\pm$ 0.02	0.89 $\pm$ 0.07	0.79
57091	Carrier	0.61 $\pm$ 0.01	1.21 $\pm$ 0.02	0.50
57217	Carrier	0.56 $\pm$ 0.02	0.64 $\pm$ 0.02	0.87
47889	Carrier	0.44 $\pm$ 0.01	0.61 $\pm$ 0.003	0.72
46146	Normal	0.81 $\pm$ 0.02	0.42 $\pm$ 0.03	1.93

Values are means $\pm$ SD of representative simplex reactions.

SMA = spinal muscular atrophy; flSMN = full-length SMN.