HHS Public Access

Author manuscript

Clin Chem. Author manuscript; available in PMC 2021 February 26.

Published in final edited form as:

Clin Chem. 2015 February ; 61(2): 412–419. doi:10.1373/clinchem.2014.231019.

Newborn Blood Spot Screening Test Using Multiplexed Real-Time PCR to Simultaneously Screen for Spinal Muscular Atrophy and Severe Combined Immunodeficiency

Jennifer L. Taylor[1,†], **Francis K. Lee**[1,†], **Golriz Khadem Yazdanpanah**[2], **John F. Staropoli**[3], **Mei Liu**[3], **John P. Carulli**[3], **Chao Sun**[3], **Steven F. Dobrowolski**[4], **W. Harry Hannon**[2], **Robert F. Vogt**[1,*]

[1]Newborn Screening and Molecular Biology Branch, Division of Laboratory Sciences, Centers for Disease Control and Prevention, Atlanta, GA

[2]Newborn Screening Translation Research Initiative, CDC Foundation, Atlanta, GA

[3]Genetics and Genomics, Biogen Idec, Cambridge, MA

[4]Department of Pathology, University of Pittsburgh Medical Center, Pittsburgh, PA

Abstract

BACKGROUND—Spinal muscular atrophy (SMA) is a motor neuron disorder caused by the absence of a functional survival of motor neuron 1, telomeric (*SMN1*) gene. Type I SMA, a lethal disease of infancy, accounts for the majority of cases. Newborn blood spot screening (NBS) to detect severe combined immunodeficiency (SCID) has been implemented in public health laboratories in the last 5 years. SCID detection is based on real-time PCR assays to measure T-cell receptor excision circles (TREC), a byproduct of T-cell development. We modified a multiplexed real-time PCR TREC assay to simultaneously determine the presence or absence of the *SMN1* gene from a dried blood spot (DBS) punch in a single reaction well.

*Address correspondence to this author at: Division of Laboratory Sciences, Mailstop F19, Centers for Disease Control and Prevention, 4770 Buford Highway, Atlanta, GA 30341. Fax 770-488-4005; rvogt@cdc.gov.

†Jennifer L. Taylor and Francis K. Lee contributed equally to the work, and both should be considered as first authors.

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Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

Authors' Disclosures or Potential Conflicts of Interest: Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest:

Employment or Leadership: J.F. Staropoli, Biogen Idec; J.P. Carulli, Biogen Idec; H. Hannon, Clinical and Laboratory Standards Institute.

Consultant or Advisory Role: None declared.

Stock Ownership: J.F. Staropoli, Biogen Idec; J.P. Carulli, Biogen Idec.

Honoraria: None declared.

Expert Testimony: None declared.

Patents: None declared.

Other Remuneration: H. Hannon, CDC Foundation, Travel SMA Meeting, DC.

METHOD—An *SMN1* assay using a locked nucleic acid probe was initially developed with cell culture and umbilical cord blood (UCB) DNA extracts, and then integrated into the TREC assay. DBS punches were placed in 96-well arrays, washed, and amplified directly using reagents specific for TREC, a reference gene [ribonuclease P/MRP 30kDa subunit (*RPP30*)], and the *SMN1* gene. The assay was tested on DBS made from UCB units and from peripheral blood samples of SMA-affected individuals and their family members.

RESULTS—DBS made from SMA-affected individuals showed no *SMN1*-specific amplification, whereas DBS made from all unaffected carriers and UCB showed *SMN1* amplification above a well-defined threshold. TREC and *RPP30* content in all DBS were within the age-adjusted expected range.

CONCLUSIONS—SMA caused by the absence of *SMN1* can be detected from the same DBS punch used to screen newborns for SCID.

Spinal muscular atrophy (SMA),[5] the most common genetic cause of death in infancy (1), is an α-motor neuron disorder caused by insufficient concentrations of the survival of motor neuron (SMN) protein. In about 95% of SMA cases, the reduction in SMN concentrations is due to deletions involving the survival of motor neuron 1, telomeric (*SMN1*)[6] gene (2). The nearly identical survival of motor neuron 2, centromeric (*SMN2*) gene, a paralog of *SMN1*, also produces SMN protein, but at much lower concentrations. *SMN2* copy numbers vary widely between individuals, ranging from complete absence to 5 or more copies per genome (3). Because some SMN protein is essential for fetal development, all babies born with SMA have at least 1 *SMN2* gene. Higher *SMN2* copy numbers in SMA patients are associated with later onset and milder disease, including juvenile onset (type III; OMIM 253400) and adult onset (type IV; OMIM 271150). However, the majority of SMA newborns become symptomatic as infants (type I; OMIM 253300) or toddlers (type II; OMIM 253550). Type 1 children will never sit unsupported, often require ventilatory support in the first year of life, and usually die before 2 years of age. Type II children can survive to early adulthood but will never walk. Cognitive abilities are not impaired by SMA and affected children are generally bright and sociable.

Several potential therapies for SMA are in development (1, 4), 2 of which are undergoing clinical trials in symptomatic children with some encouraging results (5, 6). Because of the early onset and rapid progression of infantile SMA, evaluation of these therapies in presymptomatic infants will require prompt detection. Such early detection would be possible in about 95% of SMA cases by screening newborn dried blood spots (DBS) for the homozygous absence of *SMN1* sequences around exon 7 (7, 8).

The most recent condition added to the US Recommended Uniform Screening Panel for newborn blood spot screening (NBS) is severe combined immunodeficiency (SCID) (9), a congenital disorder with severe impairment of cellular and humoral immune function due to

[5]Nonstandard abbreviations: SMA, spinal muscular atrophy; SMN, survival of motor neuron; DBS, dried blood spot; NBS, newborn blood spot screening; SCID, severe combined immunodeficiency; TREC, T-cell receptor excision circles; UCB, umbilical cord blood; Cq, quantification cycle value; LNA, locked nuclei acids.

[6]Human genes: *SMN1*, survival of motor neuron 1, telomeric; *SMN2*, survival of motor neuron 2, centromeric; *RPP30*, ribonuclease P/MRP 30kDa subunit.

a profound deficiency in T cells (10). The assay most commonly used for SCID-NBS is real-time PCR to measure T-cell receptor excision circles (TREC), extrachromosomal DNA byproducts of somatic recombination in T cells (10). SCID is the first NBS condition for which DNA analysis is the primary (first-tier) screening method. Since the initial pilot experiences in 2 US state public health laboratories (11, 12), SCID-NBS has expanded to many other state programs (13, 14) and now covers the majority of newborns in the US as well as many newborns globally (15). SCID-NBS prevents infant death through early medical intervention and is highly cost-effective (16, 17).

Because of similarities in methods, we reasoned that both SCID and SMA could be detected in the same real-time PCR reaction-well by modifying existing high-throughput TREC PCR assays to include *SMN1* genotyping. The combined assay had to be specific for *SMN1* to avoid cross-reactivity with the *SMN2* gene. Here we show that homozygous *SMN1* absence can be reliably detected from the same DBS punch used to measure TREC at minimal incremental cost.

Materials and Methods

SOURCES OF SAMPLES

DBS and DNA extracts with TREC values in the expected range for typical term newborns were made from residual excluded umbilical cord blood (UCB) units collected at the Duke University Stem Cell Laboratory. SCID-like DBS were prepared from peripheral blood containing no measurable TREC obtained from adults above age 50 years. Before spotting, the blood was depleted of mononuclear cells by layering on Histopaque (Sigma-Aldrich), centrifuging 30 min at 2100*g*, aspirating the fluid above the buffy coat, and reconstituting to 50% hematocrit with pooled serum. Immortalized B lymphocyte and fibroblast cell lines from patients with SMA (GM 23689, GM 10684, GM 03813, GM 00232, and GM 09677) and carriers (GM 23688, GM 23687, GM 03814, and GM 03815) were obtained from the Coriell Institute for Medical Research. DBS with SMA-affected or -carrier genotypes were made from residual peripheral blood samples obtained with informed consent (3) from 11 SMA-affected individuals (age range, 1–50 years) and from 15 unaffected parents (age range, 25–57 years). DBS were stored in low-permeability zip-lock bags with silica gel desiccant packs (Poly Lam Products) up to one month at room temperature, up to 6 months at 4 °C, and up to 2 years at −20 °C. The CDC laboratory staff was blinded to sample status and had no access to personal identifiers. The study was therefore classified as human subjects research for which the CDC was not engaged.

REAL-TIME PCR ASSAYS

All primers and probes (Table 1) were custom synthesized by Integrated DNA Technologies. Real-time PCR assays were conducted in PCR plates (96-well formats; Agilent Technologies) using a scanning photofluorometric thermal cycler (Stratagene MxPro 3000p). Cycle thresholds were initially determined by inspection of amplification curves and then retained in fixed positions. Quantification cycles (Cq) were reported by instrument software.

TREC QUANTIFICATION IN DBS

The real-time PCR assay was performed in situ on 2-mm discs punched from DBS samples directly into 96-well PCR arrays. After 125 μL wash buffer was added (DNA elution solution, Qiagen) to each well, the PCR array was incubated at room temperature for 15 min on a microtiter plate shaker set at 1200 rpm. The wash buffer was then removed, and 15 μL of the complete real-time PCR master mix (PerfecTa Toughmix, Quanta Biosciences) containing primers and probes (Table 1) was added to each sample well. The PCR plate was sealed with optical film and processed using the following amplification conditions: 45 °C for 3 min and 95 °C for 20 min, followed by 45 cycles of 95 °C for 15s and 60 °C for 1 min. A reagent blank and a blank filter paper punch (no-template control) were included in the analytical runs.

SMN1 ANALYSIS IN DNA EXTRACTS

DNA was extracted from cell lines or UCB samples using the Qiagen QlAamp® Mini DNA kit according to the manufacturer's protocol. DNA extracts were analyzed immediately or stored at −20 °C for up to 6 months. Extracts from tissue culture cells contained 3–50 ng/μL DNA and extracts from UCB contained 100–150 ng/uL DNA. Real-time PCR was conducted in 20-μL reaction volumes containing 5 μL of the DNA extract, a commercial real-time PCR premix (PerfecTa, Toughmix, Quanta), the *SMN1* forward primer and reverse primer, the *SMN1* locked nucleic acid (LNA) probe overlying the A>G transition at position 100 of intron 7 to distinguish *SMN1* from *SMN2*, and the *RPP30* forward primer, reverse primer, and probe (Table 1). Primers and probe sequences for *SMN1* were designed using Primer Express (Life Technologies) and confirmed for in silico specificity. Probe modification with LNA bases was designed with software from IDT Biophysics. To determine the optimal annealing temperature, reactions were carried out in a Bio-Rad Laboratories CFX96 real-time PCR instrument with the following amplification conditions: 45 °C for 3 min and 95 °C for 10 min, followed by 45 cycles of melting at 95 °C for 15 s and annealing/extension between 60 °C and 67 °C for 1 min.

Results

SMN1 ASSAY DEVELOPMENT

Prototype assays were initially explored using extracted DNA from cell lines of patients with SMA, cell lines of SMA carriers, and UCB samples. We first used a temperature gradient to determine the optimal PCR annealing temperature for maximal specificity to discriminate *SMN1* and *SMN2* amplification. The results (Fig. 1) showed increasing discrimination between the patients and unaffected controls with increased annealing temperature. At 65 °C, no *SMN1* amplicon was detected by the LNA probe in patients with SMA, whereas healthy individuals and carriers showed clear amplification. This annealing temperature of 65 °C provided the highest analytical specificity and was chosen for all subsequent experiments.

The working parameters for a duplex assay that included *SMN1* and a genomic sequence of *RPP30* as an internal control reference for PCR amplification were then optimized. This duplex assay was applied to DNA extracts prepared from commercially available cell lines

from 5 SMA patients and 4 parental carriers as well as cellular DNA from 5 UCB samples. Results (Fig. 2) demonstrated a 100% concordance with the clinical status of the donors. No amplification was seen with DNA extracted from any of the SMA patient cell lines, indicating no detectable cross-reactivity with *SMN2*. In contrast, DNA extracted from cell lines from the unaffected carriers and UCB produced robust amplification, with Cq values ranging from 18 to 25 cycles. *RPP30* in all samples showed amplification in the expected Cq range.

Having established the working conditions for the *SMN1* assay, we incorporated it into the in situ DBS assay for TREC with the annealing temperature raised to 65 °C. Reference DBS samples from a SCID-like positive control with no TREC, a positive control from an SMA infant with no *SMN1*, and a normal control from a UCB sample showed clear discrimination between the amplification patterns of the corresponding targets (Figs. 3). Varying the SMN primer concentration from 50 nmol/L to 900 nmol/L exerted no effect on the other multiplexed targets (TREC and *RPP30*; see Fig. 1 in Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol61/issue2). Therefore primer limitation was not necessary, and the maximum primer concentration (900 nmol/L) in the range tested was used for both forward and reverse *SMN1* primers in all subsequent experiments.

To determine whether the inclusion of the *SMN1* target and the higher (65 °C) annealing temperature would alter the TREC assay, we first compared results on DNA extracts (see online Supplemental Fig. 2). The TREC Cq values were highly correlated ($r^2 = 0.87$) and showed no significant difference between the original duplex (TREC and *RPP30* at 60 °C annealing temperature) and the modified triplex assay ($P > 0.14$ by paired *t*-test). Next we tested 150 DBS made from UCB with both the original duplex assay and the modified triplex assay. All samples showed the typical amplification curves expected for TREC, *SMN1*, and *RPP30*. TREC Cq values obtained by the in situ DBS triplex assay showed a slight decrease (mean 0.4 Cq) compared to those from the duplex assay but were consistent with the overall ranges for term newborns reported by public health newborn screening programs (11–14). The Cq values for *SMN1* showed a near-gaussian distribution (Fig. 4A). Cq values for *SMN1* and *RPP30* (Fig. 4B) were significantly correlated ($r^2 = 0.66$, $Sy/x = 0.47$, $P < 0.01$), suggesting that the variation between samples was due mostly to differences in leukocyte content and thus in the total amount of genomic DNA.

VALIDATION IN CLINICAL SAMPLES

The clinical validity of the *TREC-RPP30-SMN1* triplex assay was examined in a double-blind testing of DBS made from 26 blood samples from SMA patients and their carrier parents. This sample set was analyzed independently at the 2 collaborating laboratories (CDC and Biogen Idec) using the triplex in situ DBS method. The *SMN1* categorical genotypes obtained by both laboratories were identical and showed 100% concordance with the clinical status of the patient (Table 2). All samples showed typical amplification of the internal reference control *RPP30*, and the TREC Cq values were within the expected range for the age of the donor. Reagent blank and blank filter paper punches (no template controls) showed no amplification for any of the 3 targets.

Discussion

The early onset and precipitous clinical course of infantile SMA make it a prime target for early detection through NBS (7). Compared with other conditions currently recommended for NBS in the US (18), SMA has a high birth prevalence, ranking just below the top 3 of 29 NBS primary target conditions presently on the Recommended Uniform Screening Panel (congenital hypothyroidism, cystic fibrosis, and hemoglobinopathy). As with SCID, early detection of SMA is likely to provide the best opportunity for effective treatment. With multiple therapeutics currently in clinical trials, and more in development (1, 3, 19, 20), NBS laboratories are in an ideal position to facilitate presymptomatic intervention that could maximize the benefits of potential therapies.

Over the last 2 decades, the complex biology and natural history of SMA have been largely delineated (2). Although several factors influence pathogenesis, about 95% of diagnosed SMA cases are caused by the homozygous absence of a functional *SMN1* gene through either deletion or gene conversion. Various approaches to detecting the *SMN1* null genotype have been developed, and some have been validated in DBS samples, including real-time PCR (8), high-resolution melting (21), and microbead suspension arrays (22).

Two major considerations determined our approach to developing an NBS assay for SMA. First, it would be preferable to use an assay platform that is well established in public health newborn screening. Although genotype-based sickle cell disease screening had been performed previously (23), until recently none of the primary screening assays used in public health NBS programs used molecular DNA methods. The incorporation of a DNA-based assay has been prompted by the recommended inclusion of SCID-NBS, which is based on measuring TREC (24). The preferred platform for measuring TREC is real-time PCR, and this technology is now routinely used in screening the majority of newborns in the US for SCID. Second, it would be advantageous to multiplex the *SMN1* assay with an existing routine NBS assay, thereby assuring its robustness and minimizing the incremental cost for detecting SMA. We therefore explored the possibility that *SMN1* genotyping could be multiplexed with the TREC assay developed at CDC, which is used to characterize DBS reference materials for global distribution to participants in the CDC Newborn Screening Quality Assurance Program (25).

The main challenge in developing a PCR assay for *SMN1* gene absence is caused by the near identity of the *SMN1* and *SMN2* gene sequences. Conventional real-time PCR probes to any targeted *SMN1* sequence could cross-react with the corresponding *SMN2* sequence and produce false amplification signals when the *SMN1* sequence is absent, as in an affected SMA sample. Other investigators have previously reported successful reduction of cross-reacting fluorescent signals by using unlabeled *SMN2* probes as a blocker, in combination with a labeled *SMN1* probe with a minor grove binder group at the 3′ end. We opted for an alternative approach to achieve the required analytical specificity by increasing the stringency of probe hybridization using a novel LNA probe. LNA oligonucleotides increase assay specificity through restriction of the ribose conformation in the oligonucleotide backbone, allowing the use of higher annealing temperatures. These shorter probes are known to improve the ability to discern single-nucleotide polymorphisms (26), such as those

that distinguish *SMN1* from *SMN2*. LNA probes therefore simultaneously increase both the specificity and sensitivity of the assay.

Another important concern in multiplexing PCR is the potential for different amplicons to compete for PCR reagents. Since TREC is present in a much lower concentration than *SMN1*, the effect of competition would be a decreased amplification for TREC. A serial dilution series for the *SMN1* primers was analyzed to explore this possibility and showed no effect on the other multiplexed targets (TREC and *RPP30*). However, with DNA extracts of normal UCB samples and carrier cell lines, higher primer concentrations did increase the plateau fluorescence for *SMN1*, increasing visual discrimination from the baseline fluorescence of SMN1-absent SMA patients.

In addition to familiar instrumentation and multiplexing capability, another factor in developing an approach to SMA-NBS was the high prevalence of SMA carriers. In our assay, both normal and carrier *SMN1* genotypes showed robust *SMN1* amplification, and the difference in Cq was not sufficiently precise for reliable carrier identification. In contrast, all of the homozygous *SMN1*-negative samples failed to cross the cycle threshold, and they were clearly discriminated from samples with one or more *SMN1* copies.

The determination of *SMN2* copy number is an essential second-tier assay for following up screen-positive samples. It is the most informative prognostic marker and will guide selection criteria for clinical trials. *SMN2* copy number may be determined by real-time qPCR (27) as well as other methods (28). Ideally, public health NBS laboratories could perform a single second-tier assay that would both confirm the absence of *SMN1* and quantify the *SMN2* copy number, thereby identifying newborns at high risk for infantile onset. Preliminary studies in our laboratories suggest that digital droplet PCR (29) will also be a useful platform for independent confirmation and characterization of SMA screen-positive samples.

The rapid and increasingly widespread implementation of SCID-NBS has ushered real-time PCR technology into the repertoire of NBS laboratories. Since its inception in 2008, SCID-NBS has been implemented in 25 US public health NBS programs, collectively identifying SCID at twice the previously estimated birth prevalence and achieving high survival rates in treated infants (11). This experience strongly suggests that SMA-NBS will be technically feasible and cost-efficient.

In conclusion, we developed a multiplexed real-time PCR assay to simultaneously measure TREC and screen for the absence of *SMN1* in a single reaction-well. The addition of *SMN1* genotyping to the TREC assay does not require new equipment or any changes in sample processing or the overall testing procedure. The additional reagent and supply costs beyond those of the current TREC assay are limited to the *SMN1* primers and probe, which amount to less than 5 cents per test. The assay allows clear identification of the *SMN1* null genotype without quantifying copy number, thereby avoiding carrier detection. NBS programs screening for SCID will require evidence of effective presymptomatic intervention in newborns with SMA before combining the 2 tests into routine screening. However, as soon as an effective therapy becomes available, SMA-NBS could be readily implemented alone or

in combination with TREC measurements by public health programs already using real-time PCR to screen for SCID.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Research Funding: CDC Foundation through an agreement with Biogen-Idec; G.K. Yazdanpanah, Biogen Idec through the CDC Foundation.

Role of Sponsor: The funding organizations played a role in the design of study, choice of enrolled patients, review and interpretation of data, and preparation and final approval of manuscript.

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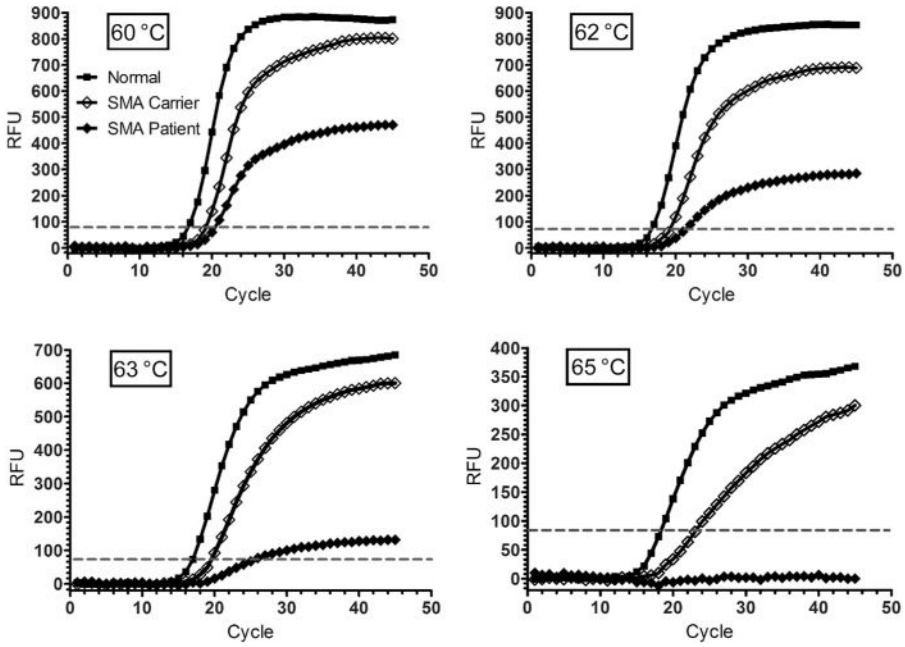

Fig. 1.

Seven temperatures (60 °C to 67 °C) were tested to determine the appropriate annealing temperature for the *SMN1* gene; results are shown for 4 selected temperatures. The dashed grey horizontal line denotes the fluorescence threshold for positive results and Cq determination. RFU, relative fluorescence units.

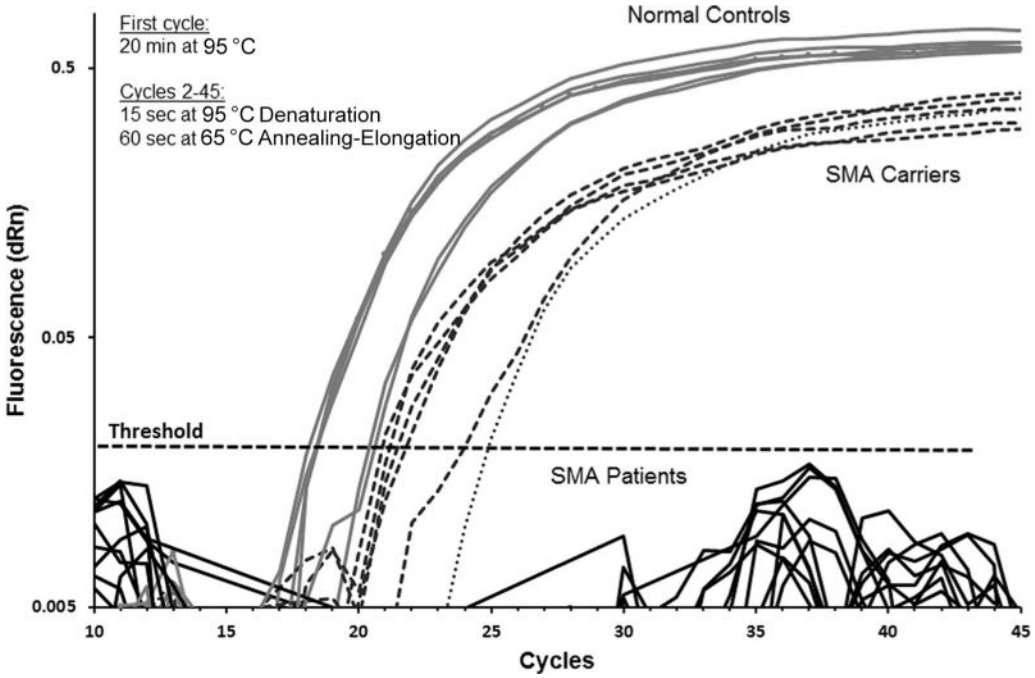

Fig. 2.

PCR amplification curves from DNA extracts of 5 randomly selected umbilical cord blood samples (normal controls), 6 cell lines from SMA parental carriers, and 5 cell lines from SMA patients with homozygous *SMN1* deletion. dRn, Δ normalized reporter.

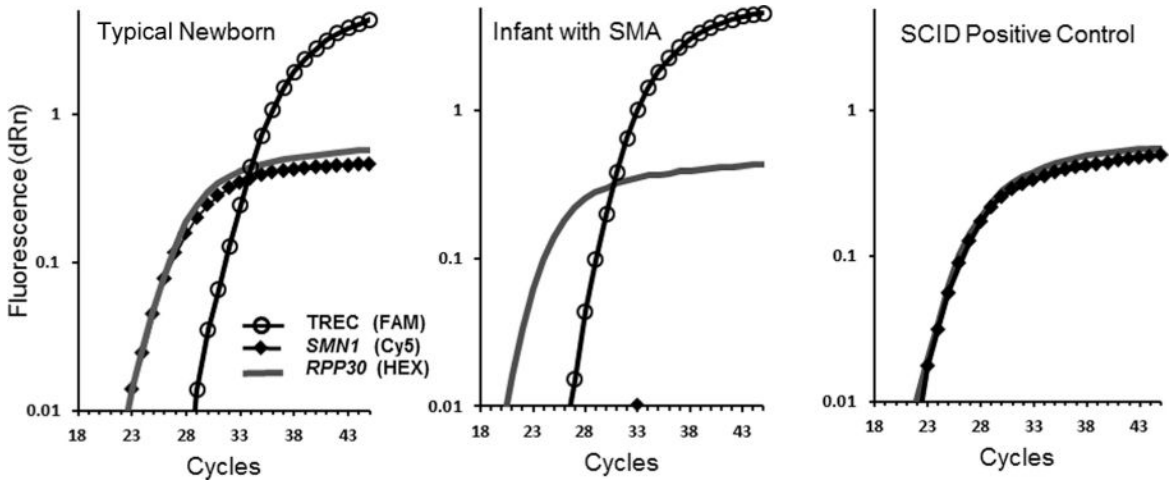

Fig. 3. Triplex TREC-SMN1-RPP30 DBS real-time PCR amplification curves

DNA targets were amplified on each DBS punch without DNA extraction. *RPP30* (present in all samples); *SMN1* (absent in SMA infant); TREC (absent in SCID-positive control). dRn, Δ normalized reporter; FAM, 6-carboxyfluorescein; HEX, β-hexosaminidase; Cy5, cyanine 5.

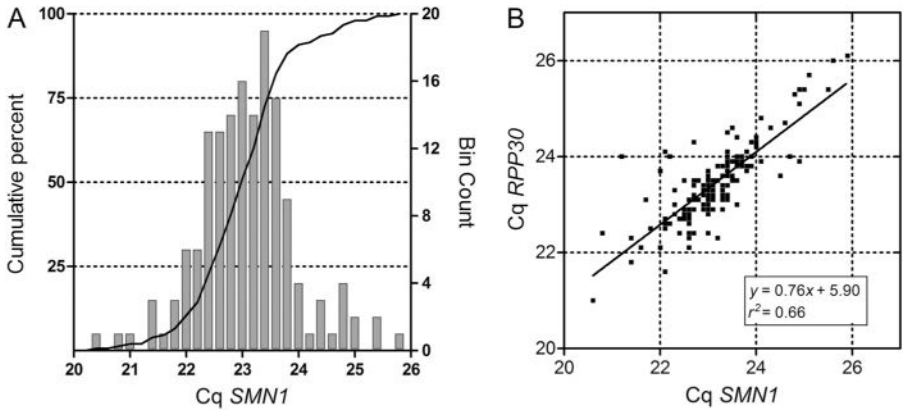

Fig. 4.

(A) Distribution of *SMN1* Cq results on dried blood spots made from 150 umbilical cord blood samples analyzed at the CDC laboratory. (B) Correlation between Cq results for *SMN1* and *RRP30* ($r = 0.66$, $P < 0.01$).

Table 1

PCR primers and probes for the triplex real-time PCR assay to amplify sequences in the δRec-ΨJα signal joint (TREC) and in *RPP30* and *SMN1*.

Target/reagent	Sequence	Concentration (nmol/L)[a]
TREC		
Forward primer	5′-TTT GTA AAG GTG CCC ACT CCT-3′	800
Reverse primer	5′-TAT TGC AAC TCG TGA GAA CGG TGA AT-3′	800
Probe	5-FAM/CGGTGATGCATAGGCACCT/Iowa Black quencher-3′	120
RPP30		
Forward primer	5′-TTT GGA CCT GCG AGC G-3′	60
Reverse primer	5′-GAG CGG CTG TCT CCA CAA GT-3′	150
Probe	5′-HEX/TTCTGACCTGAAGGCTCTGCGCG/Iowa Black quencher-3′	200
SMN1		
Forward primer	5′-GTGGAAAACAAATGTTTTTGAACATT-3′	900
Reverse primer	5′-GTAGGGATGTAGATTAACCTTTTATCTAATAGTTT-3′	900
LNA probe[b]	5′-Cy5/CAA***C***TT***T******T***TAA***C***AT***C***T/3IAbRQSp-3′	100

[a] Final concentration in PCR reaction.

[b] Bases in bold italic font denote LNA nucleotides; the underlined nucleotide indicates the position of the A>G transition in intron 7 that distinguishes *SMN1* from *SMN2*.

Table 2

Results of blinded testing of DBS samples prepared from peripheral blood samples of 11 SMA patients and 15 parental carriers by the multiplex TREC-*SMN1-RPP30* DBS real-time PCR assay.

SMA status	SMA type	Age, years	*SMN1* result[a]	*SMN1*, Cq[b]	*RPP30*, Cq[b]	TREC, Cq[b]
Patient	III	4	Absent	No Cq	23.8	30.2
Patient	III	2	Absent	No Cq	24.2	31.7
Patient	II	50	Absent	No Cq	25.1	34.6
Patient	III	3	Absent	No Cq	23.5	29.6
Patient	II	1	Absent	No Cq	24.5	30.0
Patient	II	22	Absent	No Cq	23.7	30.4
Patient	III	13	Absent	No Cq	23.2	31.3
Patient	III	3	Absent	No Cq	23.2	29.5
Patient	II	1	Absent	No Cq	21.6	28.7
Patient	III	4	Absent	No Cq	22.6	28.5
Patient	III	2	Absent	No Cq	22.5	29.1
Carrier[c]	NA[d]	45	Present	24.2	23.3	34.3
Carrier	NA	33	Present	26.9	25.0	34.3
Carrier	NA	34	Present	25.6	24.8	33.9
Carrier	NA	29	Present	25.0	23.8	34.0
Carrier	NA	32	Present	24.2	23.2	34.4
Carrier	NA	43	Present	23.3	22.2	34.8
Carrier	NA	43	Present	22.7	21.9	33.0
Carrier	NA	41	Present	23.0	22.4	35.0
Carrier	NA	57	Present	25.5	24.9	34.4
Carrier	NA	48	Present	22.7	22.3	35.3
Carrier	NA	48	Present	22.5	22.8	34.4
Carrier	NA	44	Present	25.8	25.1	36.7
Carrier	NA	35	Present	21.1	21.1	31.3
Carrier	NA	33	Present	22.6	22.6	No Cq

SMA status	SMA type	Age, years	*SMN1* result[a]	*SMN1*, Cq[b]	*RPP30*, Cq[b]	TREC, Cq[b]
Carrier	NA	25	Present	21.8	22.0	31.8

[a] Concordant categorical results from CDC and Biogen-Idec laboratories.

[b] Cq results from CDC laboratory.

[c] Unaffected parent of SMA patient.

[d] NA, not applicable.