A Multiplexed Real-time PCR Assay to Detect SCID and *SMN1* Homozygous Exon 7 Deletion, and A Droplet Digital PCR Assay to Assess *SMN2* Copy Numbers, in Newborn Screening for Spinal Muscular Atrophy

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Spinal muscular atrophy (SMA) is one of the most common lethal recessive genetic conditions, with an incidence of 1 in 11,000 births. The condition is associated with significant motor disability, respiratory and nutritional compromise, and death in infancy or childhood in more than 50% of affected children. Infants with homozygous deletion of *SMN1* and 2 *SMN2* copies typically manifest the most severe infantile form of SMA, SMA type I. However, symptom onset and disease severity in infants and children with 3 or more copies of *SMN2* is more variable. The recent development of successful medical and gene therapies has sparked an interest in early identification of affected children to provide more effective care. In 2017, FDA approved a novel treatment that could be used in the neonatal period. In 2018, the Advisory Committee on Heritable Disorders in Newborns and Children (ACHDNC) recommended expanding the Recommended Uniform Screening Panel (RUSP) to include SMA caused by homozygous deletion of exon 7 in *SMN1*, and the Secretary of Health and Human Services has accepted the recommendation. Here we report our experience in developing and validating a real-time PCR assay to simultaneously screen for SMA and severe combined immunodeficiency (SCID). Moreover, we have further developed and validated a droplet digital PCR (ddPCR) assay to assess the *SMN2* copy numbers.

Materials and Methods

DNA Isolation A 3.2 mm dried blood spot (DBS) punch from each individual undergoes DNA isolation process using either a laboratory developed buffer or Extracta™ DBS (Quanta Bio), a DNA isolution based on the same laboratory developed buffer . Briefly, each 3.2 mm DBS punch is incubated with 54 µL of Extracta™ DBS at 96˚C for 25 minutes after it was washed once with 90 µL of the same solution.

***SMN1* Homozygous Deletion Detection** The multiplex real-time PCR assay includes T-cell receptorexcision circle (TREC), *SMN1* and *RPP30* (**Figure 1**) In a 20 µL reaction mixture, there are 1X Quanta Multiplex Toughmix, TREC primers (300 nM), SMN primers (46.875 nM), *RPP30* primers (25 nM), TREC probes (150 nM), *SMN1* probes (56.25 nM), *RPP30* probes (75 nM), *SMN2* blockers (56.25 nM), and 6 µL of DNA extract. The real-time PCR conditions are 94°C for 5 minutes, 40 cycles of denaturation at 94°C for 15 seconds, annealing at 60°C for 33 seconds, and extension at 68°C for 40 seconds. The real-time PCR assay identifies the absence of exon 7 in the *SMN1* gene while simultaneously evaluating the copy number of the TREC. Additionally, the amplification of a reference gene, *RPP30*, was included in the assay as a quality/quantity indicator of DNA isolated from the 3.2 mm dried blood NBS specimens.

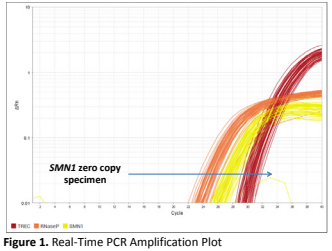

Figure 1. Real-Time PCR Amplification Plot

***SMN2* Copy Number Assessment by ddPCR Assay** We used blood spots from a well-characterized cohort of subjects with SMA, SMA carriers, and control subjects enrolled in the Newborn Screening Translational Research Network (NBSTRN) Longitudinal Pediatric Data Resource (LDPR) study. Subjects were diagnosed with type 0, I, II, III or IV SMA and had 2, 3, 4 or more than 4 *SMN2* copies. Samples from these subjects were used to validate the accuracy, reproducibility and clinical validity of this assay.

In our laboratory-developed duplex (*SMN1* and *RPP30*) ddPCR assay, the primers and probes are custom synthesized (Integrated DNA Technologies). A total 20 µL of ddPCR reaction mixture contains ddPCR Supermix (Bio-Rad), *Hae*III (0.25 U), *SMN1* primers (900 nM) and probe (250 nM), *RPP30* primers (900 nM) and probe (250 nM), *SMN1* blocker (125 nM), and 4 µL of DNA extract. Twenty µL of each reaction mixture is loaded into a sample well of a DG8™ Cartridge for QX200 Droplet Generator (Bio-Rad) followed by 70 µL of Droplet Generation Oil for Probes (Bio-Rad). After droplet generation, plates are resealed and placed in a thermal cycler for amplification. The end point PCR conditions are 95°C for 10 minutes, 40 cycles of denaturation at 94°C for 30 seconds, annealing and extension at 55°C for 1 minute, and a final step at 98°C for 10 minutes. Plates are stored at 10°C until read on a QX200 Droplet Reader (Bio-Rad).

The sealed ddPCR plate is placed into a QX200 Droplet Reader (Bio-Rad). Two PCR amplified targets, *SMN1* and *RPP30*, are viewed in a 2-D plot in which channel 1 fluorescence (FAM, *SMN1*) is plotted against channel 2 fluorescence (HEX, *RPP30*) for each droplet. Because the DNA and assay reagent distribution into droplets is random, droplets cluster into four groups: *SMN2-/RPP30-*, *SMN2+/RPP30-*, *SMN2-/RPP30+*, and *SMN2+/RPP30+*. QuantaSoft™ software measures the number of positive and negative droplets for each fluorescence in each sample, and then fits the fraction of positive droplets to a Poisson algorithm for absolute *SMN2* and *RPP30* copy numbers (**Figure 2**). QuantaSoft™ also provides *SMN2* copy numbers calculated as the fractional abundance of each target relative to *RPP30* (**Figure 3**).

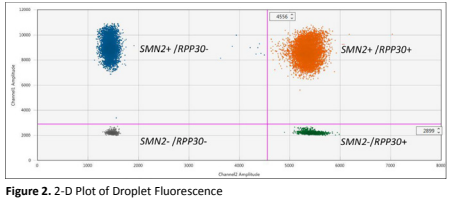

Figure 2. 2-D Plot of Droplet Fluorescence

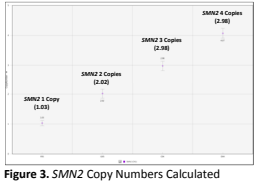

Figure 3. *SMN2* Copy Numbers Calculated as Fraction Abundance Related to *RPP30*

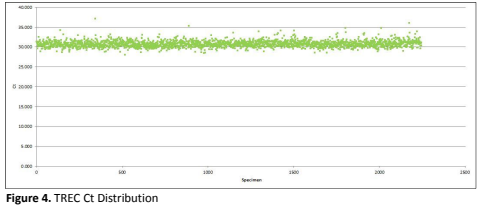

Figure 4. TREC Ct Distribution

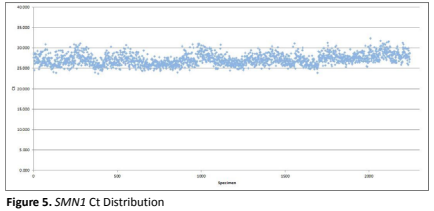

Figure 5. *SMN1* Ct Distribution

Results and Conclusion

A set of 2239 de-identified residual dried blood routine NBS specimens were used to assess T-cell receptor excision circles (TREC) population distribution and appropriate screening cutoff. The median cycle threshold (Ct) of TREC was 30.75 with the multiple of median at 1.0 (**Figure 4**). The median Ct of *SMN1* on the same set of specimen was 26.996, ranging from 23.688 to 32.376 (**Figure 5**). All seven previously confirmed *SMN1* zero dried blood specimens were tested, and had no amplification. The ddPCR assay results are summarized in **Table 1**.

We successfully developed and validated a real-time PCR assay to simultaneously screen for SMA and SCID. The additional cost for SMA screening test is minimal because it is multiplexed with an existing screening assay used to identify SCID infants. We also successfully developed and validated a droplet digital PCR (ddPCR) assay to assess the *SMN2* copy numbers. Because *SMN2* copy number is a major modifier of SMA disease where the higher *SMN2* copy numbers are associated with later onset and milder phenotype, identifying infants with 2 or 3 *SMN2* copies and treating at the earliest possible time point is critical to ensure the best outcomes. The comprehensive approach of newborn screening for SMA, that includes "just in time" knowledge of *SMN2* copy numbers in newborns who have homozygous deletion of exon 7 in *SMN1*, will facilitate early clinical follow-up, family counseling, and treatment planning.

Table 1. ddPCR Assay Performance Evaluation

	Reference		ddPCR Assay	
Clinical Status	***SMN1***	***SMN2***	***SMN1***	***SMN2***
Unaffected	2	0	2	0
	2	2	2	2
	2	1	2	1
	2	0	2	0
	2	2	2	2
	2	0	2	0
Carrier	1	2	1	2
	1	2	1	2
	1	2	1	2
	1	3	1	3
	1	3	1	3
SMA Type 1	0	2	0	2
SMA Type 2	0	3	0	3
	0	3	0	3
SMA Type 3	0	3	0	3
	0	>4	0	3
	0	>4	0	4
	0	>4	0	4
	0	>4	0	4