A qPCR Assay for Newborn Screening of Spinal Muscular Atrophy (SMA)

Zhili Lin, Sara Smith, Yang Wang, Kent Moore, Madhuri Hedge.
Revvity Omics, Pittsburgh, PA, USA

1 Abstract

SMA is the most common neurodegenerative disease in childhood with an incidence of 1 in 6,000 to 1 in 10,000. The clinical features of SMA include severe, progressive muscle weakness and hypotonia. In 2016, a pharmacological treatment of SMA was approved by FDA. Recommendation of addition of SMA to the Recommended Uniform Screening Panel (RUSP) has been submitted by the Advisory Committee on Heritable Disorders in Newborns and Children, prompting the need for a rapid but highly accurate screening test for early identification of SMA in order to facilitate timely intervention. It is critical to develop an assay which is not only cost effective, but also can be performed using dried blood spot (DBS) as this is the existing workflow that is used in the analysis of 50 disorders currently included in the NBS program.

SMA is caused by deleterious changes in the SMN1 gene, with a deletion of exon 7 being the most common pathogenic event. Homozygous deletion of exon 7 can be found in approximately 95% of SMA cases, whereas the other 5% are compound heterozygous of this deletion. Detection of the exon 7 deletion in the SMN1 gene is difficult due to the presence of the highly homologous SMN2 gene. Although mutations within SMN1 are the primary mechanism of SMA, it has since been determined that the presence of SMN2 can be used for severity prediction. Patients with a higher SMN2 copy number have been found to have reduced disease severity, as SMN2 can produce some SMN protein. Therefore, it is highly desired to develop a primary screening approach for the identification of homozygous deletion of exon 7 and a second tier assay for determination of SMN2 copy number.

We have developed a qPCR assay for the detection of homozygous deletion of exon 7 of the SMN1 gene using DNA extracted from a single 3.2 mm DBS punch. The qPCR assay is a Taqman^{R} assay using allele specific Locked Nucleic Acid (LNA^{R}) probes for both the SMN1 and SMN2 genes. The amplification of a reference gene, RPP30, was included in the assay as a quality/quantity indicator of DNA isolated from DBS. In addition, the $2^{-\Delta\Delta CT}$ method was evaluated as an alternative approach to determining SMN2 copy number. The method involves calculating relative gene expression of samples compared to a reference material with an established copy number. Although the $2^{-\Delta\Delta CT}$ calculation has several limitations, it may offer a more economical approach to SMN2 copy number determination.

2 Materials and Methods

DNA Extraction from Dried Blood Spots

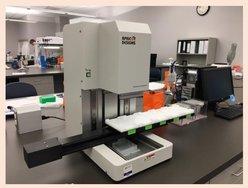
1. Punch 3.2mm dried blood spots into a 96-well plate.
2. 100 µl of Quantabio Extracta buffer was added to each well using a Apricot Liquid dispenser.
3. Incubation at room temperature for 15min.
4. Removal of Extracta buffer.
5. 50 µl of Extracta buffer was added, plate was sealed and incubated at 100ºC for 20min.

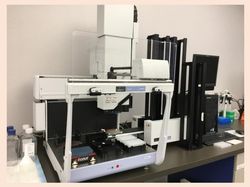
PCR Primers and Probes

Target	Primer/ Probe	Sequence	PCR Mix
SMN1, SMN2	SMN For	CTTGTGAAACAAAATGCTTTTTAACATCCAT	0.8µM
	SMN Rev	GAATGTGAGCACCTTCCTTCTTTTT	0.8µM
	SMN1 Probe	HEX/AGG+GTT+T+C+A+GAC/3IAbRQSp	1µM
	SMN2 Probe	AG+G+GTT+T+T+A+GAC/3IAbRQSp/	0.8µM
Rnase P	RPP30 For	TTTGGACCTGCGAGCG	0.2µM
	RPP30 Rev	GAGCGGCTGTCTCCACAAGT	0.2µM
	RPP30 Probe	Cy5/TTCTGACCTGAAGGCTCTGCGCG/3IAbRQSp/	0.4µM

PCR Reaction

1. PCR was setup using a Revvity Janus liquid handler.
2. A 384-well PCR plate was used.
3. Reaction was run on a Roche LightCycler 480 instrument





3 Results

The performance of this assay was demonstrated on ~1000 DNA samples isolated from putative normal newborn DBS. A total of 15 DBS reference samples from SMA patients were also included in the study.

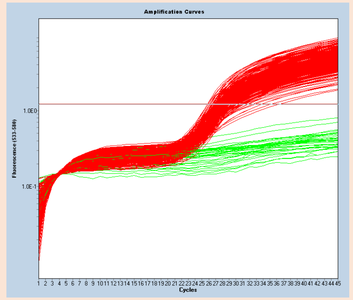


Figure 1. Amplification curves of SMN1 gene. Samples below threshold are all true positives, blanks, and SMA positive controls. All 15 SMA patients were detected correctly.

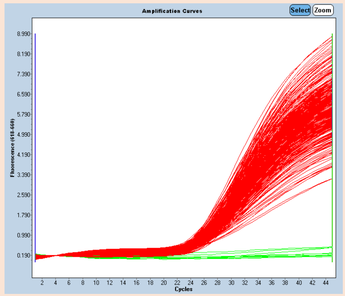


Figure 2. Amplification curves of RNase P gene. Samples in Green are blanks.

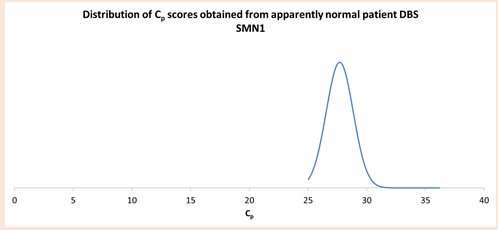


Figure 3. A Cp value is the cycle at which a significant increase in fluorescence is 1st detected. The mean Cp values of the SMN1 gene of the 1,000 normal DBS is 27.7.

$$\Delta\Delta C_T = \Delta C_{T,sample} - \Delta C_{T,control}$$

$$\Delta C_{T,sample} = (C_T\ of\ SMN1\ in\ sample) - (C_T\ of\ RNase\ P\ in\ sample)$$

$$\Delta C_{T,control} = (C_T\ of\ SMN1\ in\ control) - (C_T\ of\ RNase\ P\ in\ control)$$

$$2^{-\Delta\Delta C_T} = expression\ fold\ change$$

Figure 4. Equation used for the calculation of the SMN2 gene copy number for the 2nd tier assay.

ID	SMN2 copy # reported previously	SMN2 copy # Estimated by qPCR
SMA-031	4	4
SMA-032	4	4
SMA-035	3	4
SMA-037	2	3
SMA-041	4	4
SMA-042	4	4
SMA-045	4	4
SMA-050	3	2
SMA-060	3	3
SMA-063	3	3
SMA-069	3	2
SMA-082	3	2
SMA-086	3	3
SMA-104	3	3
SMA-105	3	3

Figure 5. SMN2 copy numbers determined by qPCR.

4 Conclusion

We have developed and validated a high throughput newborn screening assay for the detection of SMA caused by homozygous deletion of exon 7 of the SMN1 gene. However, the 2nd tier assay needs to be improved for accurate determination of SMN2 copy numbers.

5 References

1. Gerstel-Thompson JL, Wilkey JF, Baptiste JC, Navas JS, Pai SY, Pass KA, Eaton RB, Comeau AM; Clin Chem. 2010;56(9):1466-74.
2. Maranda B, Fan L, Soucy JF, Simard L, Mitchell GA; Clin. Biochem. 2012; 45:88–91.
3. Taylor JL, Lee FK, Yazdanpanah GK, Staropoli JF, Liu M, Carulli JP, Sun C, Dobrowolski SF, Hannon WH, Vogt RF.; Clin Chem. 2015;61(2):412-9.
4. Mensen A, Ochs C, Stroux A, Wittenbecher F, Szyska M, Imberti L, Fillatreau S, Uharek L, Arnold R, Dörken B, Thiel A, Scheibenbogen C, Na IK; J Transl Med. 2013; 11:188.
5. Livak KJ, Schmittgen TD; Methods 2001; 25:402-408