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FROM VALIDATION TO APPLICATION: IMPLEMENTING A COST-EFFECTIVE QPCR ASSAY FOR PRESYMPTOMATIC AND SYMPTOMATIC SMA DIAGNOSIS

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Abstract. *Spinal muscular atrophy (SMA) is a severe neuromuscular disorder caused mainly by homozygous deletion of exon 7 in the SMN1 gene. Early qualitative and quantitative diagnosis, especially in the presymptomatic stage, is essential for timely access to treatment and improved outcomes. This study focused on developing and implementing a sensitive, rapid, and cost-effective real-time PCR-based method for detecting the exon 7 deletion in SMN1. The test was first validated using 25 clinical samples, then applied in a pilot study on 300 dried blood spot samples to assess its use in presymptomatic newborn screening. The method demonstrated high sensitivity and specificity, a fast turnaround time, and suitability for both symptomatic and presymptomatic diagnosis. Compared to commercial alternatives, the custom assay proved more cost-efficient while maintaining diagnostic reliability. Despite challenges such as lack of national protocols, ethical considerations, and logistical constraints, the findings support integrating this method into routine diagnostics. Its implementation highlights the need for structured policies and infrastructure to enable equitable access to genetic screening for rare diseases like SMA.*

Keywords: cost-effectiveness, custom genetic test, real-time PCR, SMA, SMN1.

INTRODUCTION

Spinal muscular atrophy (SMA) is a group of autosomal recessive inherited neuromuscular disorders characterized by progressive degeneration of motor neurons—nerve cells located in the brainstem and spinal cord that control essential skeletal muscle activity, including speaking, walking, breathing, and swallowing. The loss of motor neurons leads to muscle weakness, atrophy and can be fatal.

Two survival motor neuron (SMN) genes are typically present in the 5q13.2 chromosomal region: *SMN1* and *SMN2*. Homozygous deletion of the telomeric copy, *SMN1* (also known as *SMNtel*), is causally linked to the development of SMA (OMIM: 253300). Although rare, with an estimated incidence of 1–6 cases per

10,000 newborns and a prevalence of 8.43 ± 0.15 per 100,000 in the Republic of Moldova, SMA is one of the most common inherited causes of childhood mortality [1][2]. The *SMN1* gene encodes the survival motor neuron protein, essential for maintaining the structure and function of motor neurons. Reduced levels of this protein lead to neuronal dysfunction and progressive muscular atrophy. The nearly identical centromeric copy, *SMN2*, functions as a pseudogene and modifies disease severity in a copy number-dependent manner. However, *SMN2* alone is insufficient to compensate for *SMN1* loss.

The most frequent causative mutation for SMA is the homozygous deletion of exon 7 in *SMN1*, accounting for approximately 95% of cases. Early identification, especially in the presymptomatic stage, is critical for accessing available therapies, as delayed diagnosis significantly decreases survival and treatment efficacy. Consequently, neonatal presymptomatic screening is vital for improving clinical outcomes in SMA [3].

Spinal muscular atrophy (SMA) is classified into four types based on motor function, with treatment now recommended for newborns lacking SMN1. However, the timing of treatment remains debated in cases with ≥4 SMN2 copies [4]. Although individually rare, such diseases collectively impact many and pose diagnostic challenges due to genetic complexity and limited resources. Since the approval of SMA therapies in 2017, early, accurate, and cost-effective genetic testing—especially presymptomatic—has become essential. This is particularly important for newborn screening programs and maternity wards with access to testing or referral systems [5][6].

The aim of this study was to develop and implement a molecular genetic diagnostic method for detecting exon 7 deletion in the SMN1 gene, associated with spinal muscular atrophy (SMA), and to evaluate the development and customization of genetic testing strategies for rare diseases, with a focus on cost-efficiency and clinical applicability.

MATERIALS AND METHODS

The molecular genetic test described in this study was developed within Human Molecular Genetics Laboratory (HMGL) with the support of institutional research projects, utilizing authorized access to the HMGL biobank of the Institute of Mother and Child (institutional agreement from 18.09.2020).

The patient cohort consisted of individuals suspected clinically of having spinal muscular atrophy (SMA) who subsequently underwent testing for the deletion associated with SMA using qualitative methods such as PCR-RFLP. A total of 25 DNA samples were included for validation of new test based on real-time PCR, of which 15 were from individuals without SMA and 10 from patients with a confirmed diagnosis of SMA, specifically the deletion of exon 7 in the *SMN1*

gene *(Table 1).*

After validation, the test was used in a pilot study aimed at applying it for presymptomatic molecular genetic screening of this disease. The pilot study included 300 dried blood spot (DBS) samples from newborns as a primary biological material.

The cost-effectiveness of the custom-developed test was evaluated by comparison with a commercially available diagnostic test. Parameters assessed included cost per diagnosis, turnaround time, and diagnostic yield. All procedures were conducted in accordance with national and institutional regulations concerning genetic testing and biobank utilization (approval no. 01-13/591 from 18.09.2020, Institute of Mother and Child).

This custom test was also employed within the framework of a doctoral research project focusing on the implementation of newborn genetic screening, approved by the university's ethics committee (approval no. 3 from 16.02.2021).

Test Principle and Methodology

The test is based on the 5' nuclease polymerase chain reaction (PCR) technique with real-time detection. It was specifically designed to identify the SMA-causing mutation in the *SMN1* gene using the amplification control gene *ALB*, from both dried blood spot (DBS) samples collected from newborns and EDTA-stabilized blood samples.

The method allows for the detection of the exon 7 deletion in the SMN1 gene—responsible for SMA—through a sensitive and cost-efficient approach using both commercially available reagents (ThermoFisher Scientific, USA) and custom-developed reagents.

Customization of the assay began with the design of specific primers using Primer-BLAST software (https://www.ncbi.nlm.nih.gov/tools/primer-blast/), followed by synthesis by BioAnalitica SRL. The primer design targeted the c.280TTC>TTT (also referenced as c.840TTC>TTT) transition, which differentiates the *SMN1* gene from its nearly identical paralog *SMN2*. This was achieved using a combined qPCR and High-Resolution Melting (HRM) technique, a molecular assay that merges the real-time quantitative amplification power of qPCR with the high sensitivity and accuracy of HRM analysis. This integrated approach offers a robust tool for detecting and characterizing genetic variations, including point mutations, while enabling qualitative and quantitative analysis of target DNA.

The qPCR+HRM method (in HMG Lab.) provides high sensitivity and specificity, allowing for point mutation detection without the need for post-PCR electrophoretic separation or labeled probes. Amplification and melting curve analysis are performed in real-time, allowing for immediate data interpretation and visualization. Real-Time PCR amplification is initiated using the PowerTrack™ SYBR Green Master Mix (Applied Biosystems™, USA) [7][8], which contains an

intercalating dye—SYBR Green—that fluoresces only in the presence of double-stranded DNA, thereby enabling detection of sequence variations. The custom primers designed for exon 7 detection in *SMN1*, the sequence of nucleotides of forward and reverse primers are included in *Innovation Act nr 562, 07 Mart 2024.* The primer design focused on the exon 7 nucleotide transition (C in *SMN1* vs. T in *SMN2*), which represents a synonymous codon change that critically affects exon splicing. This specificity is crucial, given the high sequence homology between *SMN1* and *SMN2* (only five nucleotide differences, with only one affecting transcription). Therefore, precise primer design is essential to minimize the risk of false-negative results due to nonspecific amplification.

To ensure the accuracy and reproducibility of the results, the method was validated using control samples with known SMA status. Validation was performed on the Applied Biosystems 7500 Real-Time PCR System and demonstrated 100% sensitivity and 100% specificity (*Table 1*). The complete protocol has been documented as part of *Innovation Act nr 562, from 07 Mart 2024.*

Table 1. Method validation through custom test

		SMA		
		Not affected	**SMA positive**	**Total**
Clinical diagnosis	**Not affected**	15	0	15
	SMA	0	10	10
	Total	15	10	25

RESULTS AND DISCUSSIONS

The proposed method was integrated into a molecular-genetic diagnostic process for the early identification of Spinal Muscular Atrophy (SMA) as part of a pilot study conducted at the Institute of Mother and Child Maternity Hospital during the period 2022–2024. Over this period, more than 300 informed consents and dried blood spot (DBS) samples collected on Guthrie cards were processed.

In the first phase, a pre-analytical analysis was performed, targeting the amplification of exons from *SMN1* and *ALB12* genes using real-time PCR with custom-designed primers. Melting curve interpretation was conducted using high-resolution melting (HRM) analysis. The results from this phase identified a lack of amplification of exon 7 in the *SMN1* gene and altered melting curve profiles in two samples.

Assessment of the Screening Test Sensitivity for Exon 7 Deletion in SMN1

The custom screening test employed in this study was designed to detect deletions of exon 7 in the *SMN1* gene, the genetic alteration responsible for

approximately 95% of all SMA cases. Among a cohort of 300 newborns screened, two samples tested positive and were subsequently confirmed using the MLPA (Multiplex Ligation-dependent Probe Amplification) method (*Innovation act no. 483 from 17.03.2022)* as a gold standard for diagnosis. This result indicates a 100% detection rate for this specific mutation. Accordingly: Analytical sensitivity of the test (for exon 7 deletion): 100%. Estimated clinical sensitivity (for detection of all SMA forms, including those caused by rare *SMN1* variants): ~95%. This discrepancy reflects the fact that approximately 5% of clinically diagnosed SMA cases are due to other genetic variants (e.g., point mutations, gene conversions) that are not detectable through this type of molecular screening. The method was further validated through comparison with a commercial real-time PCR kit *NeoNat SCID-SMA Multiplex* targeting SMA-associated genes (*SMN1* and *SMN2*) and an internal control gene (*β-Globin*). (Labsystems Diagnostics, ref no. 8100411) (*Table 2*) [9].

Table 2. Comparative results of applying genetic screening tests for SMA using both customized and commercial tests

No.	No. of probes	Test Type	Gene / Exon Analyzed	Positive / Negative Results	Interpre-ation	Analytical Sensitivity	Clinical Sensitivity	Specificity	Internal Validation
1	300	Customized HMGL	SMN1 / exon 7	2 / 298	2 positives for SMA. 298 negatives	100%	95%	100%	ALB exon 12 – 300/300 ✓
2	75	Commercial (Neo Nat SCID-SMA)	SMN1 / exon 7	1 / 74	1 positive for SMA. 74 negatives	100%	95%	100%	β-globin – 75/75 ✓

Cost-Effectiveness Evaluation. As part of the optimization of the neonatal screening protocol for Spinal Muscular Atrophy (SMA), a comparative cost-effectiveness analysis was performed between a custom laboratory-developed method and a commercial multiplex assay designed for the detection of exon 7 deletions in the *SMN1* gene.

The custom method (HMGL) is based on SYBR Green intercalating dye in Real-Time PCR, comprising two reactions per sample for the target gene (*SMN1*) and one for the internal control gene (*ALB*), enabling analysis of 32 samples per 96-well plate. DNA is extracted from DBS samples or blood on EDTA, and the entire workflow (extraction, amplification, and interpretation) is completed within approximately 4 hours. Both the sensitivity and specificity of this method are 100%.

In contrast, the NeoNat SCID-SMA Multiplex Real-Time PCR kit is a comprehensive commercial assay that includes all necessary reagents (including for DNA extraction) and allows for multiplex detection of mutations (*Table 3*).

Table 3. Comparative table – Cost per round (32 samples)

Parameter	Custom Test (HMGL)	Commercial Test (NeoNat SCID-SMA)
Total reactions/round	96 reactions (32 samples in duplicate + controls)	53 reactions (32 samples + controls)
Total cost/round (MDL)	~3052	~7485
Cost/sample (MDL)	~95.3	~233.9
Sensitivity/ Specificity	100% / 100%	100% / 100%
Platform used	7500 Applied Biosystems	7500 Applied Biosystems

However, each kit is optimized for 75 clinical samples, and partial usage for a smaller batch of 32 samples results in significantly higher per-sample costs. A financial analysis of a test round involving 32 clinical samples revealed a substantial cost advantage in favor of the custom method.

Implementing a molecular diagnostic test for *SMN1* exon 7 deletion in neonatal screening revealed critical challenges, including lack of national protocols, ethical concerns, logistical barriers, and limited funding. Despite sharing infrastructure with the national PKU screening program, SMA testing required additional consent procedures, data handling strategies, and specialized communication of results. These limitations highlight the need for cost-effective, ethically sound, and scalable testing strategies for rare genetic diseases.

CONCLUSIONS

The implementation of a customized qPCR-HRM method for detecting SMN1 exon 7 deletion significantly improved diagnostic accuracy, speed, and applicability for SMA. This approach offers high sensitivity and specificity, rapid turnaround, and utility in both presymptomatic and symptomatic cases, including newborns. Its successful use highlights the need for supportive policies, ethical oversight, and infrastructure to ensure equitable access to genetic screening for rare diseases like SMA. Integration into routine diagnostics is strongly recommended, especially for early detection and carrier confirmation.

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