Feasibility of a Synthetic Dried Blood Spot Mimic for use as an External Control for Newborn Screening of Genetic Disorders

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Introduction

As more therapies for the treatment of inherited disease in newborns are developed, additional tests are added to newborn screening test menus, as identified in the Recommended Uniform Screening Panel (RUSP) in the US. Severe Combined Immunodeficiency (SCID) and Cystic Fibrosis (CF) have been part of newborn screening programs for over 9 years. In 2018, spinal muscular atrophy (SMA) was approved for inclusion in the RUSP driving the need for comprehensive, multiplexed controls in a format convenient for newborn testing laboratories. In this study, multiple genetic sequences of interest were prepared within a non-hazardous blood-like matrix and spotted to Whatman 903 cards. The stability, reproducibility of DNA recovery and general utility of this dried blood spot (DBS) card mimic for newborn screening demonstrates the use of a commercially available multiplex CF control. We further extended this DBS approach for multiplexed controls for SMA and SCID assays with a biologically-relevant *SMN1/SMN2* synthetic DNA co-formulated with different concentrations of T-cell receptor excision circle (TREC) targets. Selective detection and genotyping with both the CF and SMA/SCID DBS controls demonstrates utility of this format within newborn screening laboratories and capabilities for different genotyping controls.

Materials and Methods

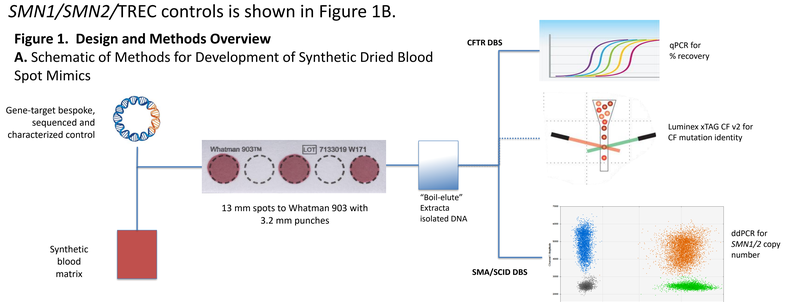
***CFTR* Dried Blood Spot Control Feasibility**: Synthetic plasmid DNAs, which included all 27 exons and intronic borders with 38 CF variants spanning the entire *CFTR* gene were suspended in a blood-like matrix spotted on Whatman 903 paper, dried overnight and extracted from 3.2mm (1/8") diameter punches using the boil-wash-elute QuantaBio DBS Extracta reagent and protocol. *CFTR* DBS eluates were tested for recovery and stability using qPCR. The CF variants were detected using the Luminex xTAG® Cystic Fibrosis v2 assay (xTAG® CF71v2 Kit).

SMA/SCID Dried Blood Spot Control Feasibility and Development: A panel of four synthetic DNA plasmids were designed *in silico* to include exons 1-8 of both *SMN1* and *SMN2* genes, intronic regions and 46 rare variants spanning the *SMN1-2* genes, a synthetic plasmid DNA including the junction site of T-cell receptor excision circle (TREC) and a reference plasmid containing a region for *RPP30*. Traceability of the synthesized clones was established by performing bi-directional, Sanger sequencing and copy number determinations via droplet digital PCR. SMA and SCID plasmid DNA were suspended in the blood-like matrix spotted on Whatman 903 paper, extracted using QuantaBio DBS Extracta from 3.2mm (1/8") diameter punches and amplified by qPCR and ddPCR.

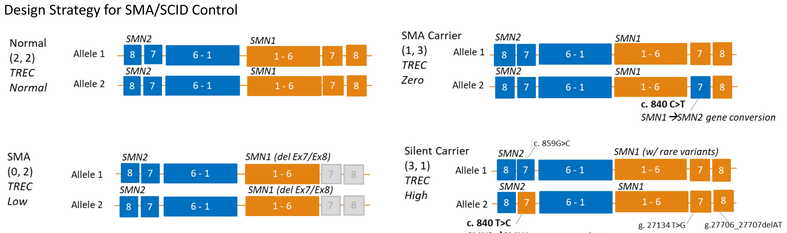
A general workflow for DBS card testing is shown in Figure 1A; a schematic formulation of the *SMN1/SMN2/*TREC controls is shown in Figure 1B.

Figure 1. Design and Methods Overview

A. Schematic of Methods for Development of Synthetic Dried Blood Spot Mimics



B. Design Strategy for SMA/SCID Control



Results

Feasibility of Synthetic Dried Blood Spot CFTR Control

Table 1. Reproducible recovery across multiple lots of Dried Blood Spot CF Controls. Four lots of DBS CF controls extracted and amplified with reproducible recovery with a % CV of less than 2 across all lots (*n=3 punches/lot*).

Starting Concentration 1.6x10^5 copies/mL	Ave Reported copies/mL	Ave Ct	SD	%CV
Lot 1	1.98x10^5	30.6	0.40	1.3
Lot 2	2.14x10^5	30.4	0.28	0.9
Lot 3	1.62x10^5	30.8	0.29	0.9
Lot 4	1.64x10^5	30.9	0.83	2.7
AVE	**1.85x10^5**	**30.7**	**0.52**	**1.7**

Table 2. Homogeneity of recovery across a single synthetic Dried Blood Spot CF control. Eight punches within a single DBS CF control show homogenous distribution with no significant variability.

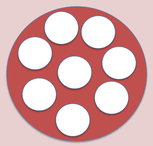
8 punch spot	Reported copies/mL	Ct	SD	%CV
	5.63x10^5	29.9		
	5.95x10^5	29.9		
	9.31x10^5	29.2		
	9.13x10^5	29.3		
	8.18x10^5	29.4		
	5.66x10^5	29.9		
	6.32x10^5	29.8		
	7.38x10^5	29.6		
AVE	**7.23x10^5**	**29.6**	**0.30**	**1.0**

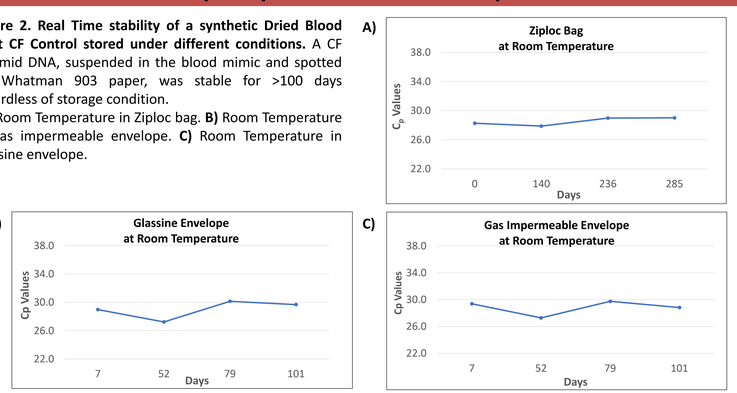
Table 3. Dried Blood Spot CF Control tested on Luminex xTAG® Cystic Fibrosis v2 Assay. Two punches tested in duplicate along with in-house MMQCI Genomic DNA on Luminex xTAG® CF71v2 Kit. All the samples were prepared according to manufacturer's protocol. The samples were read on Luminex® 200 analyzer and the output was analyzed using xTAG® Data Analysis Software. All samples passed and all variants were detected accurately.

	Correct Calls/Expected Calls	% Concordance
DBS CF Control Punch 1	28/28	100%
DBS CF Control Punch 2	28/28	100%

Stability of Synthetic Dried Blood Spot Control

Figure 2. Real Time stability of a synthetic Dried Blood Spot CF Control stored under different conditions. A CF plasmid DNA, suspended in the blood mimic and spotted on Whatman 903 paper, was stable for >100 days regardless of storage condition.

A) Room Temperature in Ziploc bag. **B)** Room Temperature in gas impermeable envelope. **C)** Room Temperature in glassine envelope.



Feasibility of Synthetic Dried Blood Spot SMA/SCID Control

Figure 3. Dried Blood Spot SMA/SCID Control evaluated by Bio-Rad *SMN1* and *SMN2* ddPCR CNV Assay[1].

A) 2-D fluorescence amplitude plot for a DBS SMA/SCID WT (2,2) Control and DBS SMA/SCID Positive (0,2) Control.

B) Correct calls for relative copy number for a DBS SMA/SCID WT (2,2) Control and DBS SMA/SCID Positive (0,2) Control.

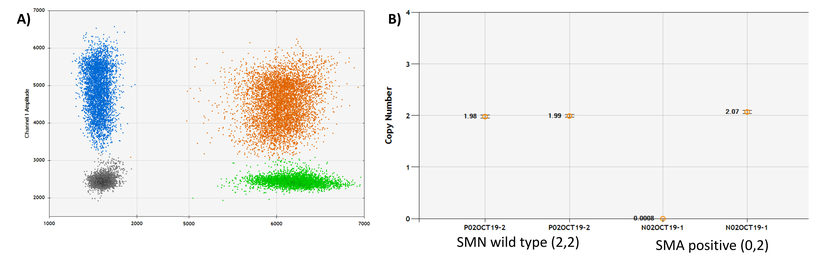


Figure 4. DBS SMA/SCID WT Control and DBS SMA/SCID Positive Control evaluated with a tri-plex qPCR for *SMN1*, TREC and *RPP30* Detection[2]. A) PCR amplification curve for detection of *SMN1* for DBS SMA/SCID WT and SMA Positive controls. **B)** PCR amplification curve for detection of TREC for DBS SMA/SCID WT (TREC normal level) and SMA Positive (TREC low level) controls and **C)** PCR amplification of *RPP30* reference gene for DBS SMA/SCID WT and SMA/SCID Positive controls.

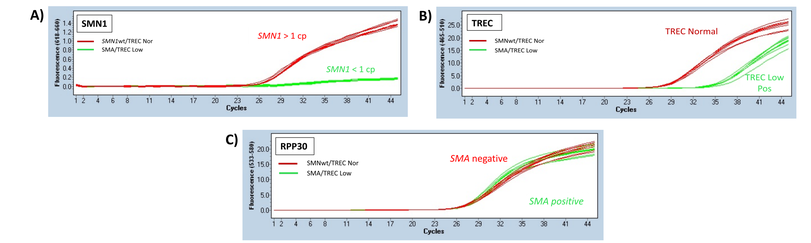


Table 4. Average C*p* values reported for DBS SMA/SCID WT Control and SMA/SCID Positive Control evaluated with a tri-plex qPCR for *SMN1*, TREC and *RPP30* Detection[2]. Eight punches each of a DBS SMA/SCID WT and SMA/SCID positive control extracted and amplified with average C*p* reported (*n=8*).

		SMN1	*TREC*	*RPP30*
Control	***Sample Type***	***C$_P$ value***	***C$_P$ value***	***C$_P$ value***
SMN wildtype/ TREC Normal	Bloodspot	26.23	28.98	27.35
SMA positive/ TREC Low Level	Bloodspot	0	35.69	27.42

Conclusions

- Synthetic DBS controls provide reproducible formulation and recovery across multiple lots with a %CV of less than 3%.
- Initial stability studies demonstrate suitability of synthetic DBS controls for potential long term storage at room temperature.
- A multiplexed DBS SMA/SCID Control yielded accurate *SMN1/2* copy numbers and TREC levels that flag normal levels and low positive reference to streamline routine monitoring of assay performance.
- Synthetic molecular multiplex DBS controls offer a viable solution as a ready to use control material for routine monitoring, verification and validation for newborn and carrier screening laboratories performing CF, SMA, SCID and other inherited diseases testing.

Acknowledgements

[1]ddPCR performed using *SMN1/SMN2* Copy Number Determination Kits (Bio-Rad, Product Code 1863500/1863503).

[2]Multiplexed primers and qPCR conditions for detection of *SMN1* and TREC were derived from J. Taylor *et al.* 2015. *Clinical Chemistry* 61:2, 412-419.