Abstract
The Recommended Uniform Screening Panel (RUSP) is a list of disorders that are recommended by the Secretary of the Department of Health and Human Services (HHS) for states to screen as part of their state universal newborn screening (NBS) programs in the US. Disorders on the RUSP are chosen based on evidence that supports the potential net benefit of screening, the availability of states to screen for the disorder, and the availability of effective treatments. In 2010, SCID screening was added to the RUSP allowing affected infants to be identified and receive treatment sooner. Similarly, evidence supporting the benefits of universal newborn screening for SMA was reviewed and the condition was added to the RUSP in July 2018.

We have developed a four-plex, real-time PCR assay to screen for SCID, XLA and SMA in DNA extracted from a single 3.2mm punch of a DBS. A simple, high-throughput, buffer DNA extraction method was developed for a Janus liquid handler that can process 384 DBS punches in four 96-well plates in just over one hour. The PCR assay identifies the absence of exon 7 in the SMN1 gene while simultaneously evaluating the copy number of T-cell receptor excision circles (TREC) and Kappa-deleting recombination excision circles (KREC) molecules. Additionally, the amplification of a reference gene, RPP30, was included in the assay as a quality/quantity indicator of DNA isolated from the DBS.

The assay performance was demonstrated on over 1000 DNA samples isolated from punches of putative normal newborn DBS. The reliability and analytical accuracy was further evaluated using DBS control and contrived positive samples. The results from this study demonstrate the potential of future molecular DBS assays and highlight how a multiplex assay could benefit newborn screening programs.

Materials and Methods
SAMPLES:
Punches from archived, presumed normal newborn samples were subjected to functional testing. Additionally, positive and negative samples were also analyzed. The SMA positive contrived samples were made by spiking GM23689 (Coriell) SMA positive cells into washed leukocyte depleted blood (LDLB) that was then spotted on Whatman™ 903 filter paper. The SMA positive samples were donated by Biogen and generated from spotting peripheral blood from SMA patients collected in EDTA tubes. Copy numbers of the SMN1 and SMN2 genes were confirmed by ddPCR. The SCID-like controls were obtained spotting peripheral blood from donors >55 years old. Other contrived samples were made by spiking plasmids into washed leukocyte depleted blood.

WORKFLOW:
The semi-automated, high-throughput system integrates PerkinElmer’s JANUS® G3 Workstations for automated DNA extraction and PCR setup and a thermocycler supporting 384-well plates.

The DNA extraction is done in a 96-well plate format using a simple isolation buffer. Then up to four extracted plates are consolidated into a single 384-well plate that undergoes PCR.

The system has the capacity of processing more than 1,500 DBS samples from sample to result in less than 8 hours with minimal hands-on time and sample tracking capability.

Results
A cohort of 1054 de-identified newborn dried blood spot samples were analyzed. Eight samples (8/1054, 0.76%) had RPP30 results above the cut-off (Ct 29) suggesting a bad sample collection or a DNA extraction failure. Two of those samples corresponded to two DBSs that had remained attached to the wall of the 96 well plate and thus failed to be properly eluted in the last step of the DNA extraction protocol. Five samples had ∆Ct values for TREC above the cut off. Five additional samples had ∆Ct values for KREC above the cut off. All the samples displayed SMN1 amplification.

The repeat rate using this assay was 1.7% (18/1054) when the cutoffs were set at the 99.5 percentile.

Functional testing
A total of twelve SMA positive samples (including SMA types I-III), one SMA carrier and 2 contrived positive SMA samples were tested. Additionally, three contrived SCID samples and one contrived XLA sample were tested. All the positive and contrived samples tested in this study were reported correctly per pre-determined cutoffs.

Acknowledgments:
We would like to thank Biogen for the SMA positive samples used in the study. This study demonstrates the potential of a multi-targeted, molecular DBS real-time PCR assay and provides a cost-effective and semi-automated solution for medium to high throughput newborn screening programs.

Summary and conclusion
The 4-plex assay presented here can effectively identify the homozygous deletion of exon 7 in the SMN1 gene and simultaneously evaluates the copy number of TREC and KREC for the newborn screening of SMA, SCID and XLA, covering two disorders already included in the RUSP. Detecting these diseases at very early ages allows for earlier treatment, possibly preventing serious damage due to disease progression.

This study demonstrates the potential of a multi-targeted, molecular DBS real-time PCR assay and provides a cost-effective and semi-automated solution for medium to high throughput newborn screening programs.