FMR1 GENOTYPING IN A COHORT OF 10,000 NEWBORN DRIED BLOOD SPOTS USING A ROBUST, SCALABLE, AND HIGH-RESOLUTION PCR ASSAY

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SUMMARY

- Fragile X Syndrome (FXS) affects roughly 1 in 4,000 males and 1 in 6,000 females, with reported carrier rates as high as 1 in 130 in females and 1 in 250 in males.
- We developed and optimized an integrated PCR-based assay system, from high-throughput sample preparation to automated software, that provides scalable, accurate and robust detection of *FMR1* repeat expansions from dried blood spots.
- The system was optimized and successfully validated using a set of 10,000 dried blood spot samples, expanding knowledge of the prevalence of *FMR1* expansions in the general population.

INTRODUCTION

Fragile X Syndrome (FXS) is a triplet repeat disorder and the most common form of inherited mental retardation and known genetic cause of autism. Carrier rates are reported to be 1:130-256 females and 1:250-810 males, and carriers are at risk for additional fragile X-associated disorders. Newborn screening (NBS) provides opportunities for behavioral therapies and other interventions at earlier ages when they may offer a greater benefit, and promises to reduce the "diagnostic odyssey" associated with FXS. In addition, clinical trials are ongoing to assess various classes of therapeutics in individuals with FXS. NBS has been favorably received by parents in prospective longitudinal studies. Accurate and cost-effective screening technologies are needed to capitalize on existing and emerging benefits associated with early detection. We describe an integrated assay approach that combines a rapid, cost-effective sample prep method, a high-throughput PCR workflow, and automated fragment-sizing analysis to assess *FMR1* genotypes in a cohort of 10,000 dried blood spots (DBS) from newborns.

METHODS

De-identified newborn DBS samples (5163 male, 4837 female) on Whatman 903 paper were obtained from the Michigan Department of Health and Human Services. The study was approved by the Institutional Review Board for the Protection of Human Research Subjects (Lansing, MI). Samples were selected across two consecutive birth years (2013 and 2014) to match the population distribution of US data available in the 2013 National Vital Statistics report distributions for race and sex[1]. Individual 3 mm DBS samples were punched into microtiter plates and DNA was eluted without formal purification using a modified version of a published high-throughput sample prep protocol[2] and an Evo 200 liquid handler (Tecan) at an estimated cost of < $0.40/sample. Eluted DNA (2 ul) was amplified directly using the AmplideX® PCR/CE *FMR1* Reagents* (Asuragen) and resolved on a 3730 DNA analyzer (Thermo Fisher) using POP7 polymer. Follow-up testing of expanded samples was performed using an orthogonal PCR method. Output FSA files were analyzed using a modified version of AmplideX® Reporter Software* (Asuragen) that automated data QC checks and reported genotype calls. Although 10,000 DBS were incorporated into the study, 616 samples were used to develop and verify the orthogonal PCR assay and analysis methods, and 70 samples were excluded for non-technical reasons. In total, only 18 samples failed in PCR, resulting in a 99.8% pass rate. The total number of newborn DBS samples used to generate *FMR1* population statistics in the study was 9296 (4500 female and 4796 male) and 13,804 total alleles.

*Research Use Only – Not For Use In Diagnostic Procedures
Preliminary research data. The performance characteristics of this assay have not yet been established.
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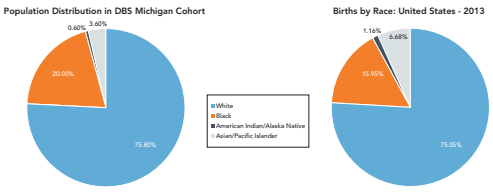

Figure 1. Population distribution of Michigan newborn DBS samples used in this study compared to US births for 2013. Samples for the Michigan cohort were selected from two birth years (2013-14) to match the approximate race distributions of US births in 2013.[1] A total of 1,067 and 1,049 male births per 1,000 female births were observed in the Michigan cohort and in 2013 per the US National Vital Statistics reports, respectively.

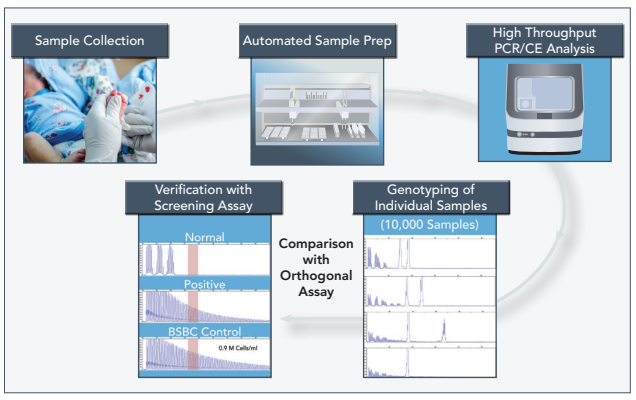

Figure 2. Workflow of the retrospective study performed using AmplideX PCR/CE *FMR1* Reagents. Chads punched from DBS cards were deposited into 96 well microtiter plates and DNA was extracted from each chad using a liquid robotic handling system and analyzed with the PCR/CE workflow. Samples flagged with CGG expansions (>44 repeats) were compared to an orthogonal PCR method. BSBC Control = Blood Spot Blood Card Control, which is a full-mutation expanded sample.

RESULTS

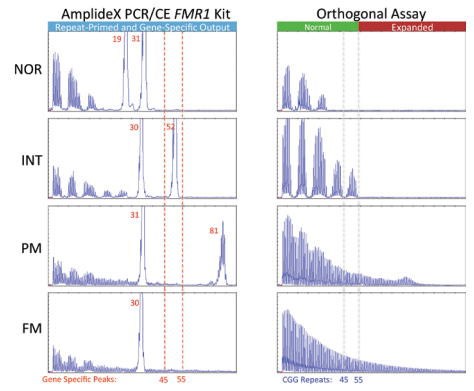

Figure 3. Comparison of primary PCR and orthogonal screening assay for *FMR1* detection in clinical samples across a range of CGG repeat sizes. Left panels show a representative example of results for AmplideX PCR/CE *FMR1* Reagents* amplification for each category: normal (<45 CGG repeats), intermediate (45-54), premutation (55-200), and full-mutation samples (>200). CGG numbers for gene-specific peaks are indicated in red font and vertical lines indicate boundaries for gene-specific peaks in the intermediate zone (45-54). Panels on the right show corresponding results for the orthogonal assay. Vertical lines indicate boundaries for the intermediate zone (45 and 55) for peaks with repeat-primed PCR profile. A set of 32 challenge samples that comprised 8 premutation and 24 full-mutation samples not from the Michigan cohort were correctly identified by both assays.

Table 1. Summary of CGG allele distribution in the Michigan (MI) cohort (final study) across gender in three categories: normal, intermediate (INT) and premutation (PM) compared with prevalence from published cohort[3]. No full-mutation samples were identified in the Michigan cohort.

Gender	MI cohort (N)	Mean (CGG)	SD	Median	Mode	MI Cohort Range (CGG)
Normal						
F	8902	29	5	29	30	6-44
M	4739	28	5	29	30	9-44
INT						
F	78	48	3	48	45	45-54
M	57	48	3	48	46	45-54
PM						
F	20	66	10	62	56	55-86
M	8	60	6	59	59	55-75

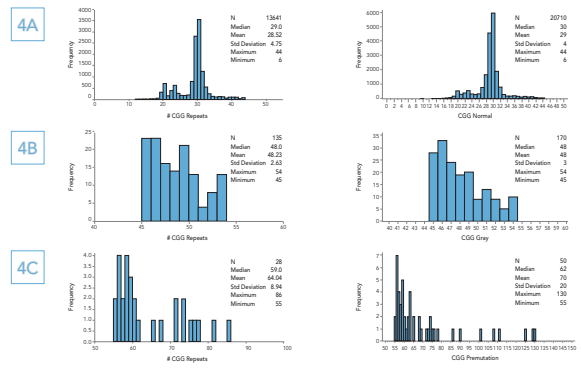

Figure 4. Size distribution of *FMR1* CGG alleles in the MI cohort, compared to published data[3]. Distribution of *FMR1* alleles are shown in the **A)** normal range (<45 repeats, n=13,641 alleles), **B)** Intermediate range (45-54 repeats, n=135 alleles), and **C)** premutation range (55-200 CGG repeats, n=28 alleles). The histograms in the second column were taken from a previously published study[3]. The boxes show the range of samples in the corresponding Michigan and published cohorts (axes have different ranges).

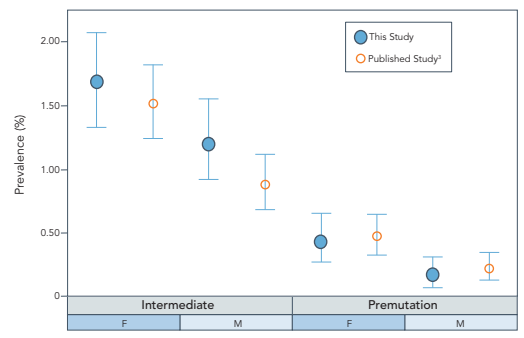

Figure 5. Prevalence across intermediate (INT) and premutation (PM) alleles compared to results from a published study[3].

CONCLUSIONS

- A high-throughput *FMR1* triplet repeat assay system was developed and validated that combined robotized, low-cost DBS processing, high-performance PCR/CE, and automated signal processing algorithms.
- *FMR1* genotypes were reliably generated using the AmplideX PCR/CE *FMR1* Reagents* with crude DBS eluates, rather than highly purified DNA.
- The PCR-based system was used to assess FMR1 expansions across 10,000 newborn DBS resulting in a prevalence of 1:60 and 1:233 for intermediate and premutation expansions respectively, in females, and 1:83 and 1:588 in males.
- No full-mutation samples were detected in this newborn cohort, although the system correctly detected 24/24 full-mutation alleles included as blinded challenge samples.
- The results confirm previously published results demonstrating a relatively high incidence of *FMR1* expansions in the general population.

References
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2. Lefterova M et al. Next-Generation Molecular Testing of Newborn Dried Blood Spots for Cystic Fibrosis. J Mol Diagn, 2016. 18(2): 267-82.
3. Tassone, F., et al., *FMR1* CGG allele size and prevalence ascertained through newborn screening in the United States. Genome Med, 2012. 4(12): p. 100.

Acknowledgments
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