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Validation of Fragile X Screening in the Newborn Population Using a Fit-for-Purpose *FMR1* PCR Assay System

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Newborn screening is designed for presymptomatic identification of serious conditions with effective early interventions. Clinical laboratories must perform prospective pilot studies to ensure that the analytical performance and workflow for a given screening test are appropriate. We assessed the potential to screen newborns for fragile X syndrome, a monogenic neurodevelopmental disorder, by establishing a customized, high-throughput PCR and analysis software system designed to detect fragile X mental retardation 1 gene repeat expansions from dried blood spots (DBSs). Assay precision, accuracy, sensitivity, and specificity were characterized across the categorical range of repeat expansions. The assay consistently resolved genotypes within three CGG repeats of reference values up to at least 137 repeats and within six repeats for larger expansions up to 200 repeats. Accuracy testing results were concordant with reference results. Full and premutation alleles were detected from subnanogram DNA inputs eluted from DBSs and from mixtures with down to 1% relative abundance of the respective expansion. Analysis of 963 deidentified newborn DBS samples identified 957 normal and 6 premutation specimens, consistent with previously published prevalence estimates. These studies demonstrate that the assay system can support high-throughput newborn screening programs. *(J Mol Diagn 2020, 22: 346–354; https://doi.org/10.1016/j.jmoldx.2019.11.002)*

Fragile X syndrome (FXS) is an X-linked disorder that leads to developmental problems, including learning disabilities and cognitive impairment (US National Library of Medicine, *https://ghr.nlm.nih.gov/condition/fragile-x-syndrome*, last accessed January 16, 2020). Of FXS cases, 99% are caused by an expansion of CGG repeats in the 5′ untranslated region of the fragile X mental retardation 1 (*FMR1*) gene.[1] Individuals with full mutation (FM) alleles have >200 repeats, whereas premutation (PM) carriers have 55 to 200 repeats. An estimated 1 in 4000 males and 1 in 8000 females are born with FXS each year.[2] FXS patients can demonstrate a wide variety of phenotypes, including speech and language impairment, hyperactivity, anxiety, and autism spectrum disorder.

There is currently no cure for FXS; however, early intervention may ameliorate developmental symptoms. Presymptomatic identification can be achieved through newborn screening (NBS) using dried blood spots (DBSs). NBS testing requires an accurate, streamlined, and high-throughput workflow that can be readily implemented in a public health laboratory. Previous pilot NBS studies for

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FXS relied on assays with design, performance, and/or operational limitations that hinder their use in routine population screening.[3–9] This article describes the validation and implementation of a screening system of *FMR1* PCR reagents, controls, and software designed for high-throughput testing of DBSs. We performed an analytical validation of this system, including accuracy, specificity, sensitivity, precision, and carryover studies, along with a population study using deidentified residual samples. These results support the potential of this system as a practical solution for high-volume FX screening to identify newborns with either the FM or the PM genotype.

Materials and Methods

Clinical and Cell Line gDNA Samples

Whole-blood specimens collected in an EDTA blood collection tube from an FM male (>200 CGG repeats), an FM female (>200 CGG repeats), and a PM female (55 to 200 CGG repeats) were obtained to make quality control (QC) DBSs from individuals evaluated at Rush University (Chicago, IL; E. Berry-Kravis) with informed consent and under institutional review board approval. DBS samples were prepared on Whatman 903 filter paper using 50 μL of whole blood pipetted within a 13-mm defined spot circle. Spots were dried within a biosafety cabinet under ambient conditions overnight before use, a common method for preparing QC DBS materials.[10,11] A normal (<54 CGG repeats) DBS and the QC unsatisfactory control (leukocyte-depleted blood), originally developed for other NBS disorders, were provided by the CDC. Accuracy studies used blinded sample sets of cell-line genomic DNA (gDNA) and DBSs with previously confirmed *FMR1* genotypes. Asuragen, Inc. (Austin, TX), supplied 20 samples of DNA from cell lines, and 18 DBS specimens were obtained as a gift from Dr. Flora Tassone (University of California, Davis). Asuragen, Inc., also provided PM female, normal male, and FM male DBS materials for the mosaicism experiments. These materials were prepared by spiking leukocyte-depleted blood with cells from corresponding cell lines into and depositing the blood onto Whatman 903 filter paper. DNA was extracted from each material and quantified, and then combined into a mixture to achieve the targeted percentage mosaicism. Finally, 963 deidentified newborn DBSs were obtained from the North Carolina State Laboratory of Public Health for the population study in accordance with an approved institutional review board protocol. DBSs were selected from consecutive specimens received at the North Carolina State Laboratory of Public Health. DBS specimens were excluded from testing because of the quality or quantity of the specimen. Examples of unacceptable specimens included blood was not completely soaked through the collection device; color was not uniform; appearance of abrasion; areas of heavy saturation; and presence of tissue fluid in the specimen.[12]

gDNA Extraction and Quantification

A 3.2-mm DBS disc was punched into each well of alternating columns of a 96-well plate using a DBS Puncher (Perkin Elmer, Waltham, MA). Each punch was washed with Extracta solution (Quantabio, Beverly, MA) to remove contaminants and inhibitors, as previously described.[13] gDNA was extracted from each DBS punch by adding 56 μL of Extracta solution to each well and then heating the solution with the DBS punch still in the well at 96°C for 25 minutes. The DNA eluate was then placed into a clean plate without the DBS punch. DNA eluate was quantified using the Qubit dsDNA HS Assay kit (Thermo Fisher Scientific, Waltham, MA), according to the manufacturer's instructions. DNA was either used immediately in a PCR or stored at −20°C.

Repeat-Primed *FMR1* PCR and CE

A mastermix solution was prepared from custom NBS reagents developed from the AmplideX PCR/capillary electrophoresis (CE) *FMR1* kit (Asuragen, Inc.) using 11.45 μL of *FMR1* NBS Amp Buffer, 1.5 μL of *FMR1* NBS Primer Mix, and 0.05 μL of GC Rich Polymerase Mix per sample.[14,15] gDNA from each sample (2 μL) was added to 13 μL of mastermix. Amplification was performed using a Veriti PCR thermal cycler (Thermo Fisher Scientific) using the following thermal cycle profile: 95°C for 5 minutes; 10 cycles of 97°C for 35 seconds, 62°C for 35 seconds, and 68°C for 4 minutes; 20 cycles of 97°C for 35 seconds, 62°C for 35 seconds, and 68°C for 4 minutes + 20 seconds/cycle; 72°C for 10 minutes; and hold at 4°C. The amplification run time was approximately 4 hours per plate with the Veriti PCR thermal cyclers. A CE mastermix was prepared by adding 11.0 μL of Hi-Di formamide (Thermo Fisher Scientific) to 2.0 μL of ROX 1000 size ladder (Asuragen, Inc.). PCR products were prepared for CE by adding 2.0 μL of PCR product to the CE mastermix. Samples were denatured at 95°C for 2 minutes. Products were analyzed on an Applied Biosystems 3730xl (Thermo Fisher Scientific) with the following injection and run protocol: injection voltage, 2.5 kV; injection time, 20 seconds at 15 kV for 2400 seconds, including oven temperature of 60°C; buffer temperature, 35°C; prerun voltage, 15 kV; prerun time, 180 seconds; first readout time, 200 milliseconds; second readout time, 200 milliseconds; voltage number of steps, 20; voltage step interval, 15 seconds; voltage tolerance, 0.6 kV; current stability, 5 μA; ramp delay, 1 second; and data delay, 60 seconds.[14]

Data Analysis and Interpretation

Genetic Analyzer data files were analyzed using a custom version of the AmplideX Reporter software version 1.2.11 that automates QC and genotype analysis for PCR/CE assays. The software performs extraction of raw trace data from *.fsa files and applies preprocessing to account for intersample and intrasample variation in signal. Sample preprocessing includes detection and elimination of signal artifacts, such as air bubbles,

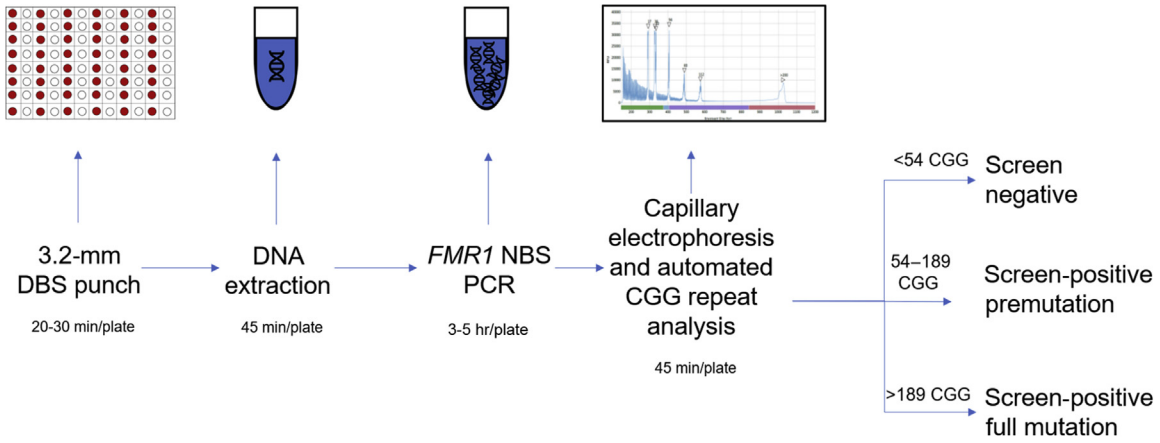

Figure 1 Screening workflow for high-throughput fragile X CGG repeat analysis. A 3.2-mm dried blood spot (DBS) punch was placed into each odd column on a PCR plate. The genomic DNA was extracted from each punch and added to the *FMR1* PCR without DNA quantification. Finally, the capillary electrophoresis was set up and analyzed on the Applied Biosystems 3730xl. Specimens with <54 CGG repeats are normal, specimens with 54 to 189 CGG repeats are screen positive for premutation, and specimens with >189 CGG repeats are screen positive for full mutation. NBS, newborn screening.

which fluoresce across all instrument channels, and channel saturation events, which result in interchannel cross talk and loss of signal resolution in the saturated channel. Automated sizing of *FMR1* gene–specific peaks is achieved via a model that uses ROX ladder peaks in conjunction with mobility correction factors determined by a batch process control with expected genotype peak sizes ranging from 18 to 200 CGG repeats; this model supports the sizing of alleles to repeats below this range. QC measures are assessed for each sample, which automatically flag samples at risk of misinterpretation on the basis of ladder integrity issues, contamination, oversaturation, or low signal. Additional batch-level QC is applied to the process and sensitivity control samples, which contain alleles that span the categorical and sensitivity bounds of the assay system.

Downstream analysis of automated CGG genotype peak calls included peaks with ≥50 relative fluorescence units. Samples were categorically classified as follows within the goals and designs for newborn screening: <54 CGG repeats, normal; 54 to 189 CGG repeats, PM; and >189 CGG repeats, FM. The classification is slightly different from the American College of Medical Genetics guidelines[2] to ensure a PM or an FM was not missed in the screening assay. Because of the clinical uncertainty of the intermediate range (45 to 54 CGG repeats), samples with <54 CGG repeats were classified as normal.

Mean, median, mode, SD, and percentage CV were calculated using R Studio version 1.2 (R Studio, Boston, MA). The analytical sensitivity data were analyzed with JMP version 14 (SAS Institute, Cary, NC).

Results

Rationale

A high-throughput workflow was designed to mimic the environment in an NBS laboratory with sufficient robustness, accuracy, and throughput to support the efficient screening of hundreds of DBSs per day (Figure 1). Critical features of the system include the following: i) compatibility with a simple and rapid DBS elution method already used by several state NBS laboratories[13]; ii) a single-well *FMR1* PCR that can genotype alleles from both male and female specimens in the primary screen; iii) a positive control comprising seven *FMR1* alleles (18, 30, 32, 56, 86, 116, and >200 CGG repeats) that verifies the detection of expanded alleles in each batch run; iv) automated analysis software that processes native instrument CE files, assesses the quality of each electropherogram, performs batch-level quality analysis through process control samples, and produces a genotype table for each set of injected samples; and v) integration of sample results within a laboratory information management system for reporting and data trending.

The system performance was evaluated according to Clinical and Laboratory Standards Institute guidelines, which are clinical laboratory best practice procedures from a consensus of global professionals that help standardize assay characteristics, such as accuracy and reproducibility,

Table 1 Repeatability of the *FMR1* PCR Screening Assay System

Sample	5-Day mean	5-Day SD	5-Day % CV
FM female	30	0.20	0.67
	>200	—	—
PM female	20	0.37	1.86
	87	0.85	0.97
	93	1.26	1.35
FM male	138	1.04	0.76
	165	2.75	1.66
	>200	—	—
Normal	20	0.33	1.65

The CGG repeat mean, SD, and % CV were calculated across the 5-day study. —, indicates SD and % CV cannot be calculated for allele sizes greater than 200; FM, full mutation; PM, premutation.

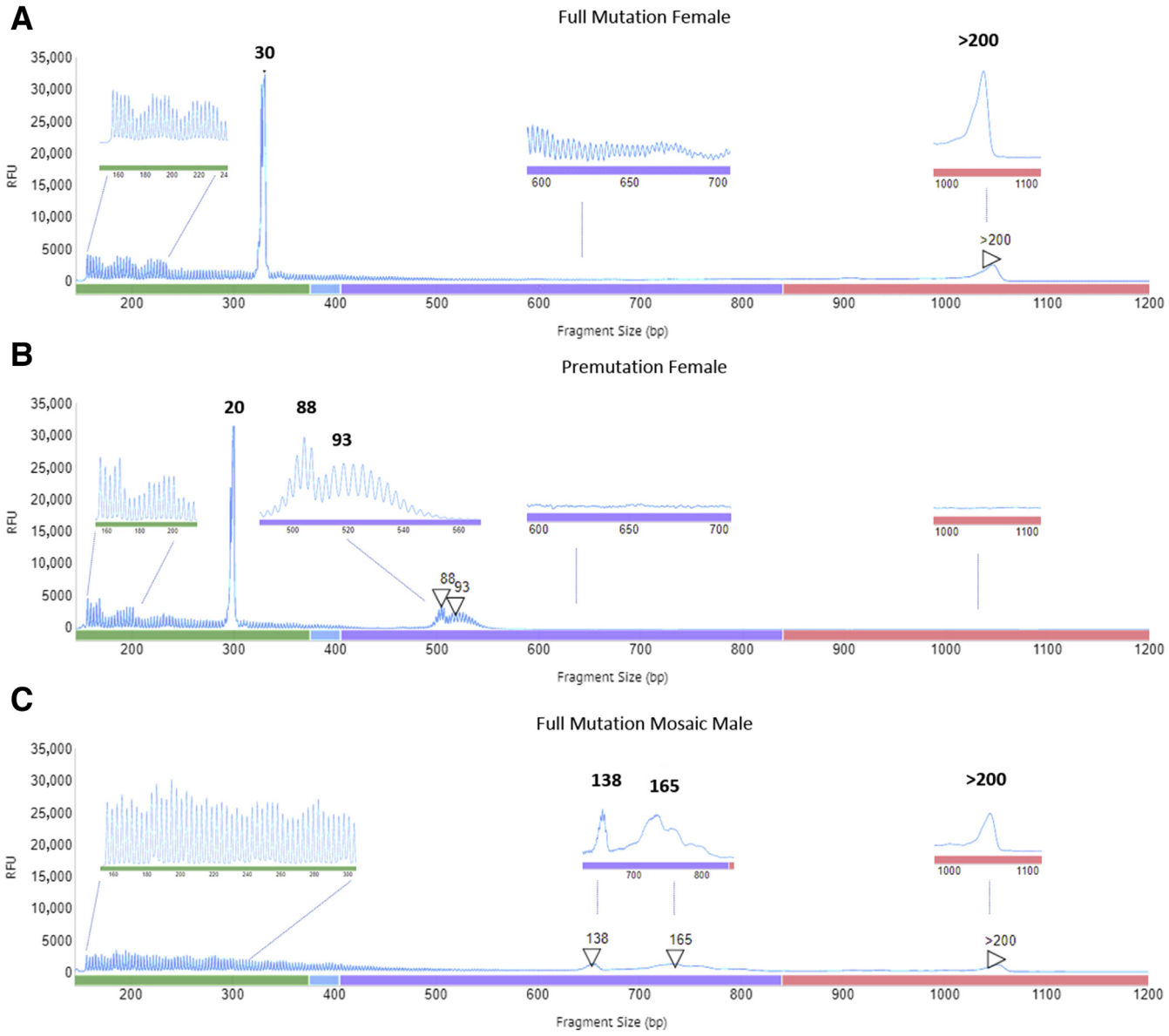

Figure 2 Representative electropherograms of PCR amplicons generated from quality control material. **A:** Full mutation female control. **B:** Premutation female control. **C:** Full mutation mosaic male control. The *x* axes represent the DNA fragment size, and the *y* axes represent the peak signal in relative fluorescence units (RFUs) of the DNA products. **Insets** in **A**, **B**, and **C** show comparisons for different levels of repeat expansion across samples.

and provide operational, performance, and quality recommendations, including those relevant to NBS tests.[16]

Preanalytical Quality Control Assessment for DBSs and Extraction

To assess the approximate concentration of DNA extracted from a 3.2-mm DBS punch, and implications for DNA input into PCR, preliminary extraction experiments were performed and the DNA quantity was obtained from eight normal control DBS punches using the Qubit dsDNA HS Assay kit. The concentration of gDNA recovered ranged from 0.475 to 0.896 ng/μL, and 260/280 absorbance ratios ranged from 1.00 to 1.67. This concentration range and the DNA purity for the subsequent PCR input produced accurate and repeatable genotypes for normal and expanded DBSs in this and subsequent studies (see below). Therefore, this rapid and robust DNA extraction was shown to be compatible with the screening system and did not require measurement or adjustment of the DNA concentration for each sample into the PCR. Consequently, DNA quantification and dilution steps previously described in the AmplideX PCR/CE *FMR1* kit were eliminated from all experiments, except for the limit of detection and mosaicism studies.[14,15]

Precision and Analytical Specificity

Precision was measured by testing each control sample (FM female, PM female, FM male, and normal) in replicates of

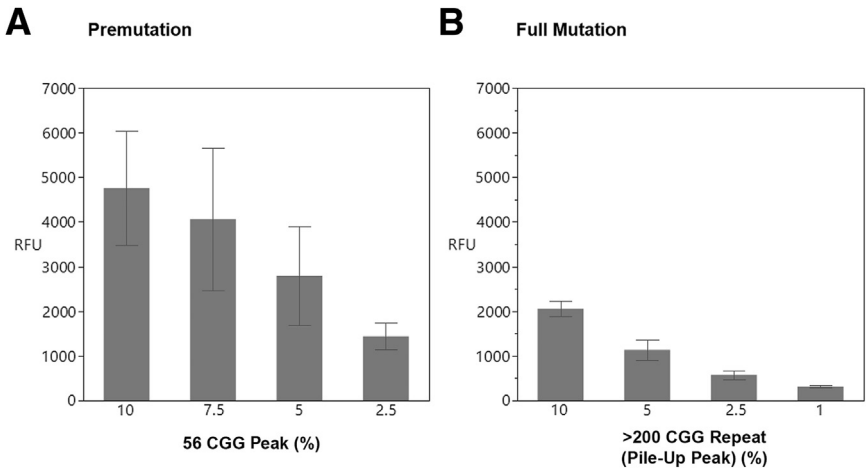

Figure 3 Analytical sensitivity for full mutation (FM) and premutation (PM) detection in the presence of normal alleles. DNA was extracted from an FM male or a PM female sample and diluted with normal DNA. **A** and **B:** The *x* axis represents percentage mass fraction of the diluted premutation (**A**) or full mutation (**B**) allele. The *y* axis represents the average signal intensity [relative fluorescence units (RFUs)] on capillary electrophoresis detected for the 56 CGG peak for the premutation sample (**A**) and the >200 peak for the full mutation sample (**B**). All samples were run in quadruplicate. Data are expressed as means ± SD.

five for 5 different test days. Two operators performed the experiments such that one operator processed plates for 4 days and the other for 1 day. Intrarun variation was tested in replicates of five on a single 96-well plate. Instrument-to-instrument variation was evaluated by comparing an identical plate on two Veriti thermal cyclers.

The CGG sizing precision between replicates for each operator, between PCR instruments, and across the 5-day testing period was <2% CV (Table 1). There was no measured difference in the CGG repeat values in the intrarun, the instrument-to-instrument, or the operator-to-operator experiments for the FM female and for normal controls. Most of the variation for each control was observed over the 5-day period. The normal allele size in all controls was ±1 CGG repeat; however, the PM alleles had a wider range of CGG repeat values without any impact on identification as a screen-positive sample (PM female, 87 ± 1 and 94 ± 3 CGG repeats; FM male, 138 ± 2 and 165 ± 6 CGG repeats). The comparatively greater variation for the 165 CGG mosaic allele in the FM male is consistent with the broad peak morphology that was observed for this expanded low-level allele (Figure 2C). Subsequent experiments using the same DNA extract stored at −20°C for up to 1 month and three freeze-thaw cycles were able to produce the same results as shown in Table 1.

To test for analytical specificity, 10 replicates each of no template control, normal, leukocyte-depleted blood, and an extraction control (filter paper with no blood) were evaluated on a single run. The no template control, leukocyte-depleted blood, and the extraction control showed no amplification, as expected, whereas normal specimens were associated with the expected repeat length.

Limit of Detection

Because the workflow was developed without a DNA quantification step, a limit of detection study was performed to further support the reliable detection of PM and FM alleles from DNA extracted from DBS specimens. Serial dilutions of DBS eluates were used to construct a standard curve. gDNA was extracted from three DBS QC materials: a PM female (0.7 ng/μL), an FM male (0.87 ng/μL), and an FM female (0.79 ng/μL). Samples were diluted to 0.5 ng/μL using the extraction solution as the diluent, and a twofold dilution was performed to achieve the following concentrations: 0.25, 0.125, 0.0625, and 0.03125 ng/μL. Two microliters was added to each *FMR1* PCR assay for a final DNA input of 1, 0.5, 0.25, 0.125, and 0.0625 ng. Each control was run in duplicate through the entire system.

Normal alleles in the PM female control (20 repeats) (Figure 2B) and FM female control (30 repeats) (Figure 2A) were detectable at all inputs. The FM allele (>200 CGG repeats) in the FM female was detectable in both runs as low as 0.125 ng; however, the FM allele in the male samples was detectable in both runs as low as 0.5 ng. PM alleles (Figure 2B) in the PM control were detectable as low as 0.125 ng in both runs. Low-abundance PM alleles were also measured in the FM male sample at inputs of 1 ng in both runs and 0.5-ng input in one run.

Analytical Sensitivity and Mosaicism

Electropherograms of expanded alleles from FM female, PM female, and FM mosaic male samples that were amplified using this NBS system manifest a multiplicity of peaks from the repeat priming of the *FMR1* 5′ untranslated region along with distinctive gene-specific peaks generated from forward and reverse primers that flank the repeat tract (Figure 2). This profile is categorically different from that of a normal sample, both in the number of peaks and their relative mobility in the electropherogram trace. As a result, samples with expansions are readily discriminated from those with normal alleles.

To determine the detectable percentage of mosaicism of abnormal alleles, FM and PM DNA eluted from 903 cards were diluted into unexpanded DNA that was also eluted

Table 2 Accuracy Results

Sample no.	Sample identifier	Reference results		Reference category	Study results		Study category
		Allele 1	Allele 2		Allele 1	Allele 2	
1	ECP014	20	—	Nor	19	—	Nor
2	ECP017	30	42	Nor	30	42	Nor
3	ECP018	20	—	Nor	19	—	Nor
4	ECP021	29	45	Nor	29	45	Nor
5	ECP024	29	30	Nor	29	—	Nor
6	ECP027	29	45	Nor	29	45	Nor
7	ECP030	30	42	Nor	30	42	Nor
8	ECP032	29	30	Nor	29	30	Nor
9	ECP025	54	—	PM	54	—	PM
10	ECP026	54	—	PM	54	—	PM
11	ECP015	91	—	PM	89	—	PM
12	ECP016	30	56	PM	30	56	PM
13	ECP022	18	114	PM	18	112	PM
14	ECP033	18	114	PM	18	112	PM
15	ECP034	30	56	PM	30	56	PM
16	ECP031	91	—	PM	89	—	PM
17	ECP013	24	>200	FM	24	>200	FM
18	ECP019	24	>200	FM	23	>200	FM
19	ECP020	>200	—	FM	>200	—	FM
20	ECP028	>200	—	FM	>200	—	FM
21	1-10-AH	23	30	Nor	23	30	Nor
22	11-10-KA	>200	—	FM	>200	—	FM
23	14-10-JT	40	—	Nor	41	—	Nor
24	15-10-BF	30	40	Nor	30	41	Nor
25	167-10-AW	52	—	Nor	51	—	Nor
26	17-10-DW	29	—	Nor	30	—	Nor
27	189-10-FR	30	50	Nor	30	49	Nor
28	20-10-JB	39	>200	FM	39	>200	FM
29	2-10-DA	30	116	PM	30	119	PM
30	211-10-FG	46	—	Nor	47	—	Nor
31	24-10-FL	83	—	PM	81	—	PM
32	27-10-TG	>200	—	FM	>200	—	FM
33	271-10-JB	30	32	Nor	30	32	Nor
34	272-10-MY	30	—	Nor	30	—	Nor
35	293-10-DC	31	51	Nor	31	50	Nor
36	3-10-LZ	30	86	PM	30	82, 86	PM
37	48-10-HC	103	—	PM	101	—	PM
38	52-10-EC	19	>200	FM	19	>200	FM

Study detected allele(s) from a single sample of extracted DNA (1 to 20) and dried blood spot specimens (21 to 38). Results were in 100% concordance with the reference laboratory results and within 5% of the expected sizing range. Category results were Nor, PM, or FM. An 82-CGG size mosaic peak was detected in addition to the reference 86-CGG peak in sample 3-10-LZ.

—, indicates second allele size was not detected; FM, full mutation; Nor, normal; PM, premutation.

from spotted material. These DNA eluates were quantified and mixed on the basis of the mass percentage ratio to yield 2.5%, 5%, 7.5%, and 10% mosaicism for the PM and 1%, 2.5%, 5%, and 10% mosaicism for the FM. The percentage input was assumed to be 50% normal and 50% expanded alleles for female samples; however, this assumption was not independently verified. Each mixture was tested in quadruplicate. The male sample with an FM allele (>200 CGG repeats) was detectable in the presence of a normal male allele (34 ± 1 CGG repeats) at 1% relative abundance in three of four replicates. The female sample with a PM allele (56 ± 1 CGG repeats) was detectable in the presence of the normal allele (34 ± 1 CGG repeats) at 2.5% abundance in all four replicates. As expected, the signal intensity for each expanded allele increased with each increasing mass fraction (Figure 3). On the basis of the quantification of the DNA in the eluates and the mixing ratio, 1% FM and 2.5% PM translated to a PCR input of approximately 0.03 ng for these expanded alleles in each case.

Accuracy

Collaborators at University of California, Davis (Flora Tassone), and Asuragen, Inc., provided 18 and 20

Table 3 Newborn DBS Premutation Sample Results

Sample no.	Size 1	Size 2	Category
89	30	57	PM
137	67	—	PM
168	20	57	PM
169	20	57	PM
241	31	55	PM
938	23	76	PM

Six premutation samples were detected of 963 DBSs. Five of the six premutation samples had two alleles, whereas sample 137 had only one allele.

—, indicates second allele size was not detected; DBS, dried blood spot; PM, premutation.

deidentified DBS and gDNA specimens, respectively, with known CGG repeats for accuracy experiments. CGG repeat results were compared between operators and with the reference laboratory that was running a clinically validated *FMR1* CGG expansion assay. All samples were run with the positive control provided in the kit and water as a no template control.

The corresponding results were in 100% categorical concordance with the reference calls for all 38 samples. The CGG repeat numbers agreed within three repeats in all cases and within a single repeat for samples with <80 CGGs (Table 2).

Reference Range

Of the 963 deidentified DBS samples tested, 1342 alleles were identified. A total of 379 specimens had two alleles and 584 had one allele. There were 957 specimens (99.4%) classified as normal, and 6 (0.6%) had PM alleles with CGG repeat values ranging from 55 to 76. Although sex and other demographic information were not collected for any samples in this study, five of the six PM samples had one normal allele in addition to the PM allele (Table 3).

The pass rate for classifying samples using the screening system for the initial test was 98.6%, and all retested samples produced acceptable results after the second PCR from the same gDNA extraction. Assay failures were limited to poor amplification, aberrant ROX ladder migration, or aberrant peak calls. Figure 4 shows the CGG repeat population distribution of the largest allele size in each sample. The mean allele size of the largest allele in each sample was 31, and the SD was 5.0 (Table 4). The mean allele size found in the entire population was 29, and the SD was 5.21. The most common allele size was 30. The normal population statistics for the study are in accordance with a previously reported newborn screening pilot study.[3]

Discussion

FXS is the most common form of inherited intellectual disability and is typically diagnosed after children present developmental delays. Presymptomatic screening and intervention mitigates the diagnostic odyssey for families and allows for expanded treatment options for children before symptoms become apparent.[17] Several published studies have described results from pilot NBS for FXS in the United States, yet each has relied on a screening assay that would be impractical to implement for routine, high-volume use in state laboratories.[3–9] For example, Saul et al[4] screened 1459

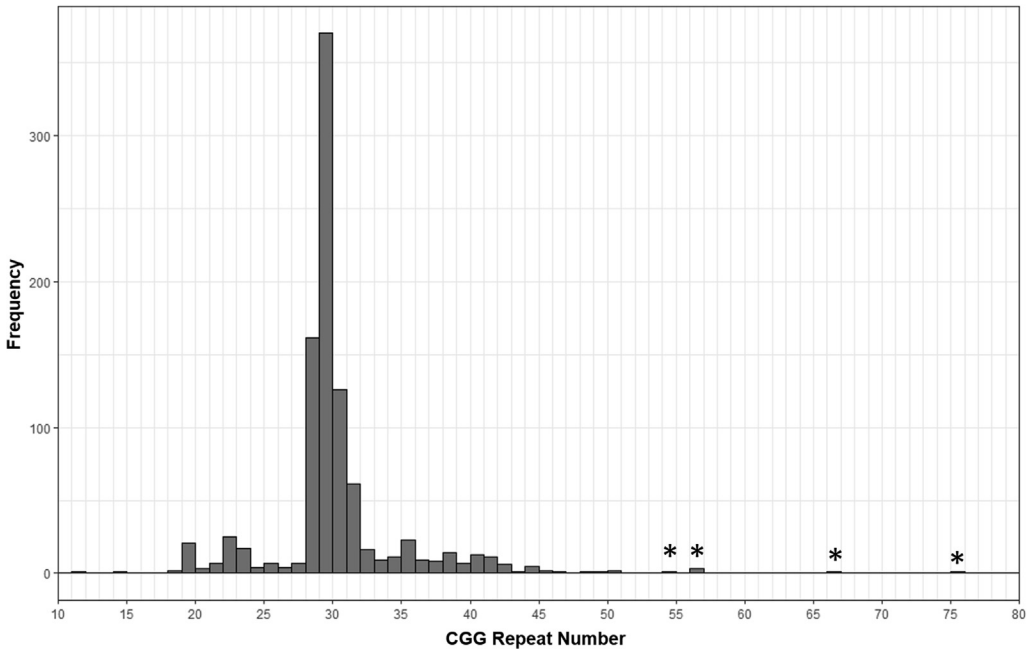

Figure 4 CGG repeat population distribution in 963 dried blood spot (DBS) specimens. The *x* axis represents the CGG repeat length of the largest allele in each sample, and the *y* axis represents the frequency of that allele in the cohort (North Carolina deidentified newborn DBS samples). Samples were categorized on the basis of the largest allele present, regardless of abundance. **Asterisks** represent peaks with allele sizes >54 CGG repeats.

Table 4 CGG Repeat Length Population Statistics

CGG repeat	Mean	Median	Mode	SD	Min	Max
Largest allele per sample	31	30	30	5.00	12	76
All population alleles	29	30	30	5.21	12	76

Statistics were calculated for the largest allele in each sample and all the alleles found in the 963 newborn screening samples that were evaluated.
Max, maximum; Min, minimum.

newborns with an *FMR1* PCR/CE assay. This method could not consistently detect expansions and relied on PCR allele dropouts to help flag abnormal alleles. Expansions in males, but not females, were the focus of the study because the assay could not be validated with female specimens.

A separate study of 36,124 newborns used methylation-sensitive PCR, but this assay was similarly limited to screening male newborns.[5] The workflow required significantly more sample preparation, including an additional procedure of bisulfite DNA treatment, multiple cleanup steps, and real-time quantitative PCR. By comparison, Tassone et al[3] used a repeat-primed *FMR1* PCR/CE method that could flag expanded alleles in both males and females to screen 14,207 newborns. Yet, this approach required two different PCR designs and reagent sets, a reflex strategy for the primary screen, and manual analysis. Several other *FMR1* gene- or protein-based technologies have been proposed for NBS,[6–8] but each has similar disadvantages in identifying the appropriate at-risk population, producing reliable results at scale, achieving the requisite sample throughput, realizing the necessary workflow efficiency/integration, and/or actualizing sex equity through screening.

Herein, we describe fit-for-purpose *FMR1* PCR reagents, software, and controls that can accurately and reproducibly quantify CGG repeat length using a high-throughput DBS testing method. In contrast to previously reported *FMR1* NBS assays, the current method is a screening system that integrates and optimizes preanalytical, analytical, and postanalytical steps to genotype alleles from both males and females in a single PCR. More important, the system identifies normal, PM, and FM CGG repeats without the need for formal DNA purification and concentration measurements, and instead uses a rapid, single-reagent DBS elution. There are several extraction protocols available that produce more purified DNA, but this one was chosen because of the lower cost and the current protocol is in use in NBS laboratories.[13]

The detection and reporting of carrier status in NBS evokes important clinical and ethical issues[18]; the system described herein allows for the reporting of such information if NBS programs decide that disclosure would be important or expected. The system also provides a standardized reagent set, a multiallele process batch control, and custom genotyping software that automatically performs quality checks, peak selection, and conversion of fragment size to CGG repeat numbers from the raw CE instrument data file.

For a genetic marker to be used for high-throughput NBS, analytical and clinical validity must be demonstrated. The analytical specificity and precision of the system was found to be well within the acceptable kit parameters and within Clinical and Laboratory Standards Institute guidelines.[16] FM alleles were detected in as little as 0.5 ng in male gDNA and 0.125 ng in female gDNA extracted from DBS QC material, although routine isolates from DBS are greater than these lower limits. Consistent with this yield of DNA, 1% to 2.5% mass fraction of FM and PM alleles was detected in a background of a normal DNA using spotted material. Detection was achieved using 1 to 2.6 ng total DNA input into PCR. This analytical sensitivity is consistent with detection of mosaic expanded alleles down to at least 10% in DBS DNA, which is the lower level of mosaic detection reported for triplet-repeat PCR in diagnostic assays.[2] Finally, despite the sensitivity of the system to detect low levels of PM or FM alleles, no evidence of carryover from expanded CGG repeat samples into blank samples was found.

The accuracy testing results were in 100% categorical concordance with reference genotypes and within three CGG repeats for alleles in the sizable range (Table 2). No false-positive or false-negative samples were found in the accuracy testing panel. The 963-sample population study yielded a 98.6% first-pass rate, and all retested samples were successfully genotyped on rerun from the same gDNA extraction. The mean allele sizes and SDs in Table 4 were comparable to the results found in Tassone et al.[3] A 0.6% PM prevalence (95% CI, 0.29%−1.35%) was observed, as expected; no FM alleles were identified.[3] Five of the six PM samples appeared to be female, although sex could not be verified because of the deidentification before testing.

Typically, CE data require time-consuming, manual analysis of electropherograms. Manual interpretation is a significant barrier to high-volume testing and is prone to user error dependent on the level of skill and training of the operator. The automated analysis module used in the *FMR1* NBS assay system enables processing of a 96-well plate of samples in <5 minutes, whereas manual analysis by conventional software solutions would require at least 30 minutes per plate. This automated data analysis would allow FXS to be incorporated into a high-throughput NBS environment that tests hundreds to thousands of specimens daily. In fact, it is calculated that a laboratory with two bench staff, along with two CE instruments with 48-capillary arrays and three thermal cyclers, could genotype >1000 DBS samples per week with the existing workflow. The batch size could be doubled by changing the punching and extraction format from 48 to 96, and combining this change with additional thermal cyclers would further expand the testing capability.

In summary, we describe and validate an integrated set of reagents, instruments, controls, and software that can reliably detect unexpanded and expanded *FMR1* alleles in a newborn population screening cohort. Infants identified through the FX screening system can be confirmed through testing in an independent diagnostic laboratory capable of

detecting *FMR1* expansions, as well as by performing other tests, such as those that determine AGG interruption status. This method may allow offering screening for carrier status, depending on parents' preferences and public health considerations. Further studies are needed to test the assumption that earlier identification can lead to better outcomes for children and a net benefit to families and to society as a whole.

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