Accurate Fragile X Triplet Repeat Sizing and Archival Storage from a Range of Clinically Accessible Biosamples

Andrew Hadd, Jon Kemppainen, Stela Filipovic-Sadic, Liangjing Chen, Jeff Houghton, Ned Sekinger, and Gary Latham

Asuragen, Inc., Austin, Texas, USA

SUMMARY POINTS

- New research method shows robust FMR1 (Fragile X Mental Retardation 1) gene amplification from multiple biosamples of the same donor
- 10 male and 10 female donors comprised of 5 matched specimens each - blood, blood spot card, buccal swab, mouthwash and hair follicle
- Successful detection by agarose gel and capillary electrophoresis and concordance across matched sample types
- Size verification by DNA sequencing and characterized standards
- Whole genome amplification from each primary DNA source

INTRODUCTION

Fragile X syndrome (FXS) is the phenotypic consequence of CGG repeat expansion in the 5' UTR of the FMR1 gene following female meiosis. This expansion is correlated with increased methylation of the gene promoter and a loss of FMR1 gene expression. While full expansion of the CGG region is associated with FXS, asymptomatic premutation alleles (55-200 CGG) are associated with ataxia in men and ovarian insufficiency in women. PCR analysis is the primary method for determining normal, intermediate and shorter pre-mutation alleles related to FXS. Our purpose was to establish robust new research procedures for sizing FMR1 alleles from a variety of specimen types including whole blood, buccal swab, mouthwash, blood spot card, and hair follicle. Additionally, we evaluated whole genome amplification (WGA) as a strategy to archive genomic DNA for FMR1 gene testing, particularly from relatively DNA-poor samples.

MATERIALS AND METHODS

Specimens were obtained from consenting, healthy donors under IRB, grouped by gender and de-identified. Specimens were collected until at least 10 male and 10 female donors were matched across the five specimen sources: whole blood (B), mouthwash (MW), cheek (C), hair follicle (H) and blood spot card (S). FMR1 Primers and GC-Rich PCR buffer prototypes were mixed with purified DNA aliquots (2 µL) or directly to dried blood spot card punches in microtiter plates. Following PCR, samples were analyzed without purification either by agarose gel electrophoresis (AGE) or by capillary electrophoresis (CE). The general workflow is shown in Figure 1. DNA sequencing results were obtained using purified PCR products, ABI BigDye 3.1 chemistry and prototype FMR1 sequencing reagents. Whole Genome Amplification (WGA) reactions were performed on 1 matched male and 1 matched female sample set (B, MW, C, H and S) following manufacturer recommended procedures.

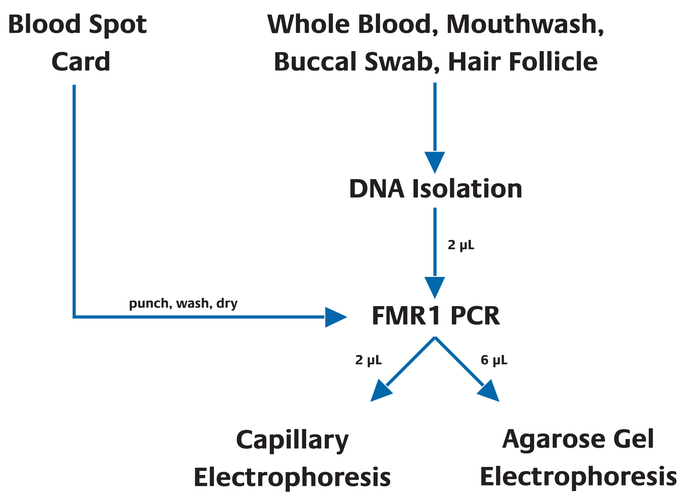

Figure 1. General FMR1 amplification and detection workflow.
Tissue specimens were collected and extracted using column-based procedures. Specimens from blood spot cards were directly added to the PCR as washed and dried 1.2 mm punches. Samples analyzed by AGE were sized using a standard 10,000 bp molecular weight ladder (L) and a 6-band process control (PC) containing templates corresponding to 21,29,31,56,118 and 198 CGG-repeats. Samples analyzed by CE were processed using GeneMapper 4.0. CGG repeat length was determined using cell-line standards referenced to DNA sequencing.

RESULTS

DNA Yield and FMR1 PCR Product Bands by Source

	Donor	Blood	MW	Cheek	Hair
Male	001	22.2	41.0	37.0	52.1
	003	24.0	34.0	5.8	16.0
	004	28.2	28.2	18.5	39.3
	005	21.8	68.1	21.2	17.9
	006	24.8	6.0	16.8	9.6
	007	19.7	60.9	17.0	12.0
	010	20.8	27.4	23.5	77.1
	011	31.7	54.7	12.8	12.4
	012	20.6	20.7	6.9	86.9
	013	16.8	5.4	7.5	12.0
Female	101	25.1	14.9	15.5	<5
	102	17.3	27.0	11.4	10.8
	104	27.7	23.1	9.8	7.8
	105	28.3	52.6	10.7	7.8
	106	35.9	41.1	5.9	102.2
	107	28.6	25.0	13.8	20.0
	108	18.1	32.8	8.4	<5
	109	22.8	19.9	5.7	27.9
	111	20.8	16.5	6.7	10.1
	112	26.2	89.1	14.8	19.2

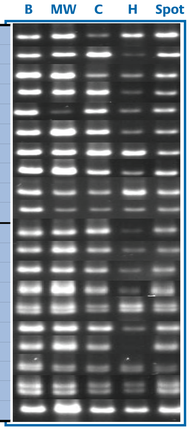

Figure 2. Comparison of purified DNA yield in ng/µL with FMR1 PCR bands on AGE.
DNA was isolated from whole blood (B), mouthwash (MW), cheek (C) and hair (H) following standard procedures. Yields of isolated DNA were obtained in ng/µL for four of the specimen types (blood spot DNA was directly added to the PCR without isolation). Samples not determined by NanoDrop are listed as <5 ng/µL (101- and 108-Hair). FMR1 PCR products were obtained as isolated bands across five sample types, blood, mouthwash, cheek and hair with blood spot card punch (1.2 mm). Equivalent sizing across sample sources was observed in each donor set except for weak or no signals for 003-, 101-, and 108-hair samples. A single band was observed in all male and in 7/10 female samples.

Effect of DNA Quantity and Source on FMR1 PCR

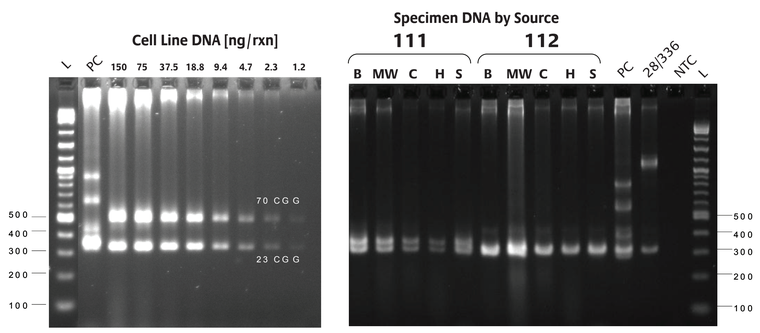

Figure 3. FMR1 PCR product bands on AGE by DNA quantity and specimen source.
A 23/70 Cell line DNA sample was titrated from 1.2 to 150 ng input into the PCR (left) along with two samples across specimen sources (right). A molecular weight ladder (L) and Process Control (PC, 21,29,31,56,118 and 198 CGG), no-template control (NTC) and a 28/336-CGG cell line sample are shown for reference. The PCR band intensity decreased proportionally to the input DNA from 75 to 1.2 ng. PCR products between donors 111 and 112 were consistent by source; two bands were observed for sample 111 with only a single band for sample 112.

Whole Genome Amplification of gDNA from Potentially Limiting Samples

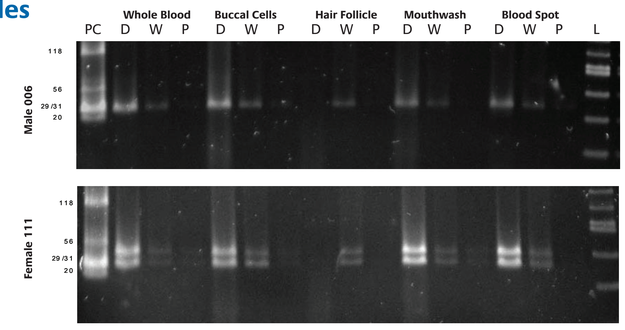

Figure 4. Whole Genome Amplification by sample source on AGE.
Samples were run as extracted DNA (D) at 20 ng per reaction, WGA products (W) normalized to 20 ng input and PCR controls (P) as dilution control of the original genomic DNA. Sample source indicated in top of figure.

Capillary Electrophoresis by Specimen Type

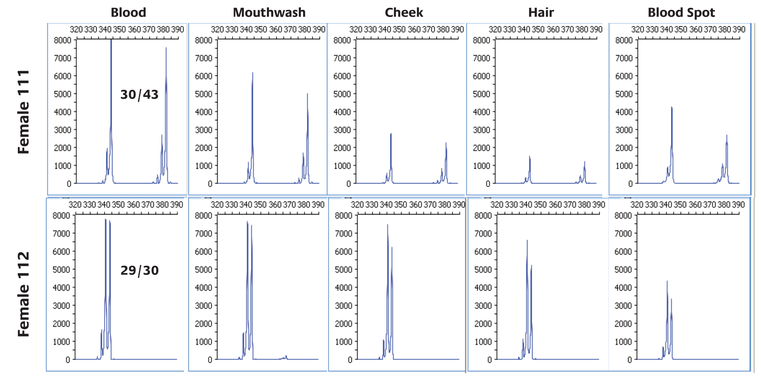

Figure 5. Effect of specimen source on peak resolution and yield.
Electropherograms of Sample 111 and 112 donor sets. PCR products were generated using FMR1 Primers and GC-Rich PCR buffer prototypes combined with 2 µL aliquot of isolated DNA or direct blood spot card punch. Following PCR, 2 µL of unpurified products were added to 13 µL of Hi-Di Formamide containing a 1000-bp ROX-labeled standard ladder. Sample sizes were within 0.5 bp for all sources of DNA across all donor sample sets. Two peaks were observed for sample 112 compared to a single band by AGE (compare to Fig. 3).

Verification of CGG Repeat Length by CE and DNA Sequencing

	Sample	CE Sizing	Sequencing	AGG
Male	001	29	29	*9,19*
	003	32	32	*10,20*
	004	20	20	*10*
	005	30	30	*10,20*
	006	29	29	*9,19*
	007	30	30	*10,20*
	010	29	29	*9,19*
	011	29	29	*10*
	012	37	37	*9,17,27*
	013	30	30	*10,20*
Female	101	29/31	29/ND	*9,10,19,20*
	102	31/36	31/ND	*10,20*
	104	30	30	*10,20*
	105	30/32	30/ND	*9,10,20,22*
	106	20/32	20/ND	*9,10*
	107	30	30	*10,20*
	108	24/30	24/ND	*10,13,20*
	109	20/29	20/ND	*9,10*
	111	30/43	30/ND	*9,10,19,20*
	112	29/30	29/ND	*9,10,19,20*

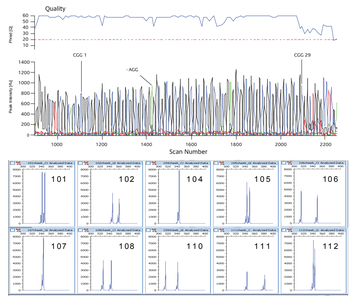

Figure 6. Comparison of CGG repeat length by CE and DNA sequencing analysis
CE sizing results were obtained by comparing size in base pairs, determined using the ROX-ladder, with CGG standards referenced to DNA sequencing. Sizes between source of DNA were equivalent across each donor set. DNA sequencing results were analyzed for the "AGCG" start and prominent thymine peak at the end of the CGG repeats for reference (top right). Intervening –AGG sequences are listed by repeat number in the table. Male samples were concordant by DNA sequencing. Female samples were concordant for the smaller CGG allele and were not determined (ND) for the second allele due to heterozygosity in the trace after the first allele. Distribution of allele sizes for the 10 female samples were obtained (bottom right). Single peak samples 104 and 107 were confirmed homozygous by Southern Blot analysis (data not shown).

CONCLUSION

- FMR1 Primers and GC-Rich PCR buffer prototypes used for successful amplification of 5 specimen sources from 20 donors, including dried blood spots.
- Equivalent CGG repeat sizes observed across specimen sources for each donor.
- CGG repeat sizes assessed by CE were concordant with DNA sequencing.
- Whole Genome Amplified samples demonstrated equivalent sizing across each sample source and improved recovery of DNA from low yield samples, and thus represent a potential method for archiving samples of limiting DNA.
- Utility of alternative non-invasive collection methods for routine Fragile X testing may provide greater flexibility in sample collection and archival storage.
- Results from these research studies support a future evaluation of clinical samples comprising pre-mutation and full mutation alleles.