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Optimization, validation and initial clinical implications of a Luminex-based immunoassay for the quantification of Fragile X Protein from Dried Blood Spots

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Abstract (no more than 350 words)

Background: Fragile X syndrome (FXS) is the most common inherited form of intellectual disability affecting 1 in 4,000 males and 1 in 6-8,000 females. FXS is caused by a trinucleotide expansion in the 5'UTR of the Fragile X Mental Retardation (*FMR1*) gene which in full mutation carriers (>200 repeats) leads to hypermethylation and transcriptional silencing of the gene and lack of expression of Fragile X Protein (FXP, formerly known as Fragile X Mental Retardation Protein, FMRP). Phenotypic presentation of FXS is highly variable, and molecular markers explaining or predicting this variability are lacking. Recent studies suggest that trace amounts of FXP can be detected even in fully methylated individuals and may have clinical relevance; however, the lack of available reproducible, sensitive assays to detect FXP in peripheral tissue makes evaluation of peripheral FXP as a source of clinical variability challenging.

Methods: We optimized a Luminex-based assay to detect FXP in dried blot spots for increased reproducibility and sensitivity by improving reagent concentrations and buffer conditions. The optimized assay was used to quantify FXP in 187 individuals (101 males, 86 females; 0-78.4 years) including 35 typically developing controls (24 males, 11 females), 103 individuals carrying full mutations (70 males, 33 females), and 49 individuals with premutations (7 males, 42 females). A subset of these individuals showed repeat number or methylation mosaicism. We investigated the clinical relevance of peripheral FXP levels by examining its relationship with general intellectual functioning in a subset of individuals with available IQ scores.

Results: We show that the optimized assay is highly reproducible and detects a wide range of FXP levels. Mosaic individuals had, on average, higher FXP levels than fully methylated individuals, and trace amounts of FXP were consistently detectable in a subset of individuals with full mutation FXS. IQ

scores were positively correlated with peripheral FXP levels in male and female individuals with full mutation FXS.

Conclusions: We demonstrate that our optimized Luminex-based assay to detect FXP is reproducible, highly sensitive, and related to the core intellectual disability phenotype. Further, our data suggest that trace amounts of FXP detectable in dried blood spots of individuals with FXS could be clinically relevant and may be used to stratify individuals with FXS for optimized treatment. Future studies are needed with larger sample sizes, evaluating FXP across development and expanded analysis of the relevance of FXP levels for behavioral and electrophysiological phenotypes in FXS.

Key words: fragile X syndrome, biomarker, molecular phenotype

Background

Fragile X Syndrome (FXS) is the most common single gene cause of autism spectrum disorder (ASD) and most common inherited cause of intellectual disability impacting 1 in 4,000 males and 1 in 6-8,000 females worldwide (1). FXS results from CGG triplet repeat expansion in the promotor region of the FMR1 gene located on the long arm of the X chromosome (2). Typically, over 200 CGG repeats result in gene methylation and transcriptional silencing of the FMR1 gene. The CGG repeats in the full mutation range are typically inherited and undergo expansion when passed from a premutation carrier mother (55-200 CGG repeats) to her child (2). As a disorder of gene silencing, FXS results from deficient production of the FMR1 gene product, fragile X protein (FXP, previously termed fragile X mental retardation protein (FMRP)) (3). FXP serves as a translational repressor impacting the expression of hundreds of proteins vital to brain function including those critical to cognitive functioning (4).

FXS is associated with a behavioral phenotype marked by high incidence of anxiety, ADHD, language and cognitive deficits among other clinical features (5-7), and with physical presentations including, but not limited to, pronounced ears, soft tissue laxity and macroorchidism in males (8). Despite commonalities in the presentation, significant variation in the behavioral phenotype does exist within FXS. Females with FXS are obligate mosaics with two X chromosomes resulting in a highly variable phenotype in girls and women ranging from no appreciable developmental impairment to significant development delay or intellectual disability. Even among males with FXS, phenotypic developmental variability is represented by functioning levels from severe to mild or even borderline intellectual/cognitive impairment (8). In part, this variation may be due to mosaicism in FXS. Repeat size mosaicism can occur when individuals have a mix of premutation and full mutation repeat alleles, whereas methylation mosaicism can occur where clinical Southern Blot and PCR testing may indicate inconsistent methylation patterns with a mix of fully and non-fully methylated FMR1 alleles regardless of CGG repeat length. Given the phenotypic and genetic variation in FXS, it is of critical importance to understand how this variation may relate to variable FXP expression in this disorder.

Evaluation of FXP expression in individuals with FXS has been challenging due to difficulties with sensitivity and specificity of available assays. In addition, there are limited studies assessing how well FXP levels in accessible peripheral cells reflect FXP levels in the brain and whether FXP in peripheral cells is associated with characteristic phenotypic features. Nevertheless, in recent years there has been considerable progress in methodology for FXP detection and measurement in peripheral tissue such as human blood, skin fibroblasts, hair follicles and buccal cells (9-14). One of the first attempts to evaluate FXP levels used immunofluorescent staining in blood lymphocytes (15). This method was subsequently used to document the significant relationship between higher FXP levels and higher cognitive functioning based on IQ scores (6, 16, 17). However, this assay did not take into account different expression levels of FXP in individual lymphocytes and thus lacked sensitivity to detect a spectrum of

92 FXP expression, which may be clinically relevant. Despite Western blot and ELISA (enzyme-linked
93 immunosorbent assay) methods providing a continuous readout that better captures the full range of
94 FXP expression, these methods are difficult to scale up (western blot) and lack quantifiability, sensitivity
95 and/or specificity (reviewed in (14)).

96 Recently, a novel highly sensitive assay was developed measuring FXP as a continuous variable
97 in peripheral blood using Luminex-based technology (18). This method is not only more sensitive to
98 detect lower values of FXP than previous methods, but also the Luminex assay is easily scalable and has
99 increased specificity due to the use of two different highly specific FXP antibodies and. Moreover, this
100 assay can be used with eluates from dried blood spots, which facilitates the potential future application
101 of this assay outside the research laboratory and into clinical settings. The FXP Luminex assay was used
102 in a recent study to measure FXP levels in 42 samples of individuals with FXS and demonstrated that
103 males with severe ID had lower FXP than males with mild or moderate ID (19). However, the lower limit
104 of FXP detection was above zero indicating difficulty differentiating “true zero” FXP expression from
105 potential low or trace level FXP levels, thus also limiting its capacity to be more clinically relevant among
106 males with full mutation.

107 Thus, given the major advantages the Luminex assay offered over previous methods to measure
108 FXP, we sought to optimize this method to improve upon the detection of FXP in peripheral blood to
109 best discern potential molecular variation in FXS. We believe a highly sensitive and reproducible FXP
110 assay will be important to new treatment development as FXP expression likely varies extensively in FXS
111 despite the single gene/single protein nature of the disorder. In appreciating molecular variation, we
112 aim to in the future use this understanding as a means to biologically subgroup persons with FXS when
113 evaluating clinical presentation, outcome, and response to potential therapeutics. Here, we report on
114 our initial work to optimize FXP detection in human blood with a focus on enhancing assay accuracy and

115 improving the lower limit of detection using the FXP assay in broad subgroups of individuals with
116 emphasis on populations of males and females with full mutation FXS.

117 **Methods**

118 **Participants**

119 We enrolled a total of 187 participants: 101 males and 86 females aged 0 – 78 years (Table 1).
120 Participants with a full mutation (70 males and 33 females) or premutation (7 males and 42 females) in
121 the FMR1 gene were recruited through the Cincinnati Fragile X Research and Treatment Center. Fragile X
122 status was confirmed at minimum by clinical Southern Blot (SB) and/or Polymerase Chain Reaction (PCR)
123 testing to confirm group assignment. Among individuals with full mutation FXS, 54 males and 29 females
124 had reliable research standard SB and PCR analysis completed at Rush University to evaluate for repeat
125 size mosaicism, methylation mosaicism, or expression of both mosaicisms. Within this subsample, 18
126 males (33.3%) exhibited methylation and/or size mosaicism and 9 females (31%) had methylation
127 mosaicism in addition to being obligate size mosaics. Control subjects (24 males and 7 females) were
128 recruited through web-based fliers from the local community and had no prior diagnosis or treatment
129 for developmental or neuropsychiatric disorders. All participants or their legal guardians gave written
130 informed consent and/or verbal assent, when appropriate. This project was approved by the CCHMC
131 IRB.

132 Table 1. Demographic Information.

	No.	Age			Fragile X Protein (FXP) Concentration (pM)			
		Average	SD	Range	Average	Median	SD	Range
Male	101	19.2	15.3	0.3 - 61.6	10.2	1.7	13.4	0.0 - 43.2
Typically Developing	24	26.0	14.9	5.5 - 60.1	28.9	27.5	6.4	18.1 - 43.2
Premutation	7	31.1	23.4	2.2 - 61.6	30.5	26.1	7.9	22.6 - 42.1
Fragile X Syndrome	70	15.7	13.2	0.3 - 45.7	1.7	0.5	2.6	0.0 - 10.3
Nonmosaic	36	18.2	14.6	0.3 - 45.7	0.6	0.3	1.2	0.0 - 6.2
Mosaic	18	14.8	13.8	0.5 - 41.5	3.8	2.6	3.2	0.0 - 10.3
Female	86	33.2	18.9	0.2 - 78.4	26.4	24.8	10.6	3.1 - 64.9
Typically Developing	11	20.9	18.9	0.2 - 63.8	31.6	27.5	13.7	16.1 - 64.9
Premutation	42	47.3	13.2	9.0 - 78.4	30.1	28.5	9.6	11.8 - 55.2
Fragile X Syndrome	33	19.4	10.3	1.2 - 42.9	19.9	20.9	7.1	3.1 - 33.8
Methylation Mosaicism	9	16.4	7.8	1.2 - 25.2	22.0	20.9	7.7	9.3 - 30.6

Blood Collection and Processing

Blood samples were collected from all participants in 2 mL Vacutainer K2EDTA tubes (BD, 36781) and inverted 10 times before processing to ensure homogeneity within the sample. 50 µL of blood was pipetted onto ID Bloodstain Cards (Whatman Bloodstain Cards, WB100014) producing at least two cards with 13 spots each from one sample collection. Cards were dried and stored with desiccant packs in low-gas-permeable bags (VWR, 89027-022) within 4-24 hours after spotting to ensure DBS stability, in accordance with dried blood spot (DBS) guidelines and published protocols (18, 19) (Figure 1).

Elution of DBS

From each card, five 6.9 mm diameter disks were prepared using a hole punch and transferred into CoStar Spin-X Centrifuge Filter Tubes (7200388). Proteins were extracted from the DBS using 333 µL of elution buffer (M-PER with salt, Antipain, Chymostatin, Protease Inhibitor) with orbital shaking overnight at room temperature. The eluates were collected after a 6-minute centrifugation at 12000xg and immediately used in the assay. 50 µL of the eluate was used per well in the assay (Figure 1).

Immunoassay Procedure

An 11-point standard curve was created using a purified GST-SR7 fusion protein obtained from the Institute for Basic Research in Developmental Disabilities (IBR). Elution buffer was used to complete a two-fold dilution of the first standard point generating a standard curve with range of 70-0.07 pM. Either 50 µL of standard or DBS extract was aliquoted into assay wells of a 96-well low protein binding plates (Greiner Bio-One, 655096). The capture antibody, mAb 6B8 (BioLegend, 834601), was concentrated according to manufacturer's instructions (Abcam, ab102778). The concentrated mAb 6B8 was coupled to Luminex Magspheres according to manufacturer's instructions and constructed at a stock concentration of 100µL antibody/all beads. Beads were diluted in assay buffer (PBS, 1% BSA, 0.05% Tween) to 80 beads/µL for use in assay. Diluted beads were added to the assay wells at a volume of 50 µL to bring the final well volume to 100 µL. Plates were then incubated at room temperature for 6 hours on a microplate shaker. A Luminex magnetic plate washer was used to manually wash the plates in assay buffer. After washing, the plates were incubated overnight in secondary detecting antibody (ab17722, Abcam) at a dilution of 1:1000 (v:v). Plates were vigorously washed and incubated at room temperature for 2 hours in signal detecting antibody (Jackson ImmunoResearch, 711-116-152). Plates were vigorously washed and resuspended in 100 µL of sheath fluid (Luminex, 40-50021). The magspheres were analyzed (in quintuplicate) on the Luminex 200 system to determine median fluorescence intensity (Figure 1).

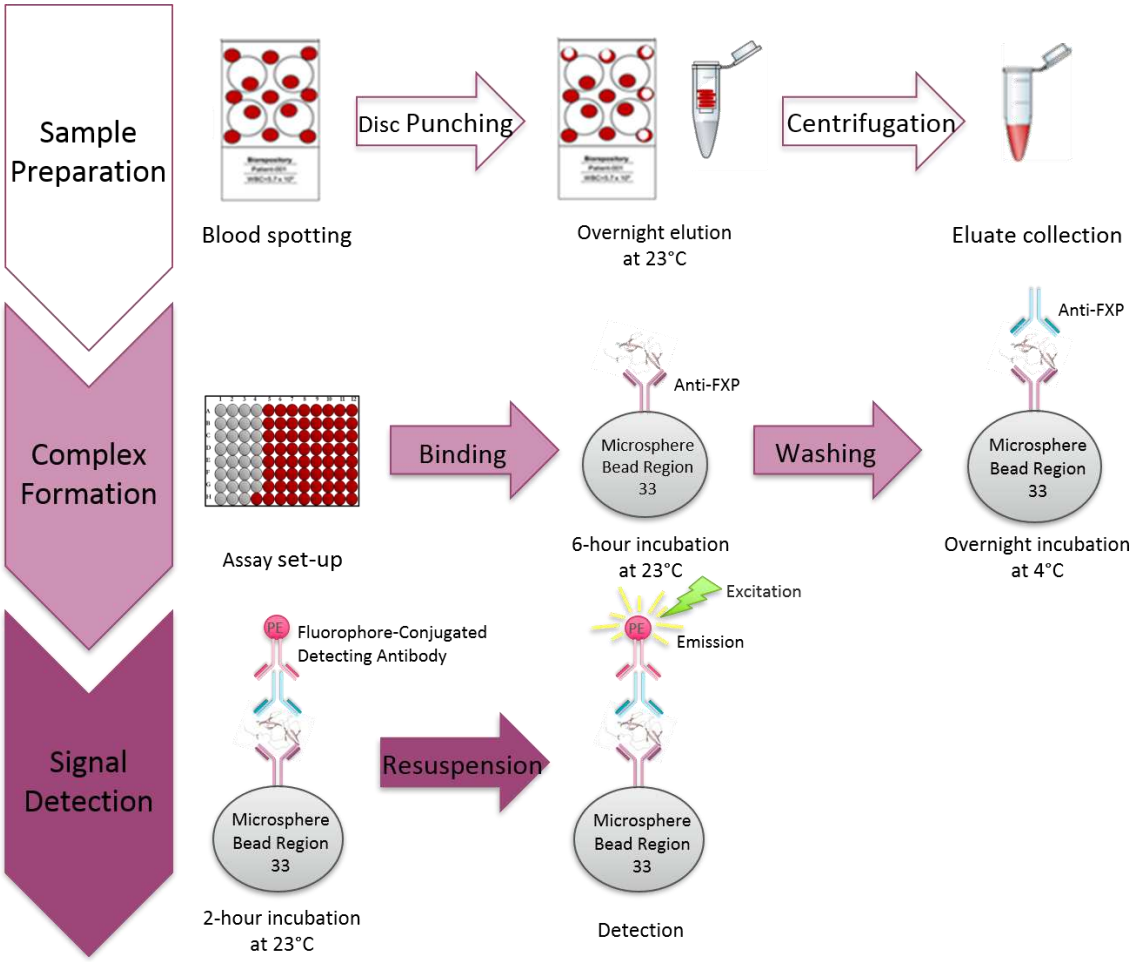

Figure 1. Experimental workflow of the optimized FXP assay.

Fragile X Protein (FXP) Quantification

To determine individual FXP concentration, BioPlex Manager Software was used to generate a standard curve of GST-SR7 concentration as a function of median fluorescence intensity. Patient samples were plotted against this curve and reported as concentration (pM) in the DBS extract. Based on the volume of blood spotted, the size of the DBS, and the elution volume, we estimate peripheral FXP circulating in the blood is approximately six times the value reported in the assay.

Individual FXP concentrations were reported as a 20% trimmed mean of the observed concentrations between the sample quintuplicates. The sample mean is prone to undue influence by

extreme observations, while the sample median is inefficient if the data are normally distributed. The 20% trimmed mean, i.e., the average of the three central values, is used as a compromise between the sample mean and sample median. Differences in protein concentration between groups were analyzed using Kruskal-Wallis tests with Dunn's multiple comparison adjustment in GraphPad Prism software. A *p*-value < 0.01 was considered significant.

Assay Reproducibility Analysis

The stability of FXP over time was analyzed using the Intra-Class Correlation Coefficient (ICC) via SAS ® software. Specifically, the agreement across time was measured using the ICC where the response, FXP, was modeled as a function of subject ID and week. The Shrout-Fleiss measure of reliability, ICC, was used where the subjects were evaluated at the various repeated measure timepoints (20). The ICC here was the same regardless of whether the weeks were treated as a fixed or random variable.

To best characterize the assay, we completed rigorous testing of its performance statistics. We measured intra-assay variability, inter-plate variability, inter-draw variability, and inter-card variability to determine an acceptable standard of variation. Previous studies (20, 21) using the Luminex-based immunoassay platform for FXP-unrelated assays have reported intrinsic variabilities and ranges of acceptable variation. Based on those reports, we defined poor variation as CV > 15%, good and acceptable variation as CV < 15%, and excellent variation as CV < 5%. For the calculation of the performance statistics, all interpolated values were considered regardless of their acceptance as a reported value (Figure 2). Intra-assay variability, or the variability between replicates, was measured and reported as an average %CV for each set of quintuplicates across all participant samples and plates.

Outlier Removal Analysis

We used the coefficient of variation (CV) as the standard measure of variability for this assay. Percent CV was determined using the observed concentration calculated via the BioPlex Manager Software [i.e.,

CV= (standard deviation (of observed mean quintuplicates)/mean (of observed mean quintuplicates)) *100]. Due to intrinsic variability within any biological test, we set a threshold for acceptable variation within the quintuplicates from one sample as a CV < 15%. This threshold is within the range of the performance statistics of this assay and in alignment with similar methods (20, 21). If upon the initial assay, a sample set of quintuplicates produced an observed concentration CV < 15%, the result was accepted and reported as the 20% trimmed mean of the observed concentration. If the results of the initial assay indicated CV ≥ 15%, the assay was repeated with a new set of 5 DBS from the original DBS card, if possible. If less than 5 DBS were available, the largest amount of available DBS was used. If the results of the repeated assay indicated CV < 15%, the results of the second assay were accepted and reported as the 20% trimmed mean of the observed concentration. If the CV ≥ 15% for both the initial and repeated assays, both sets of quintuplicates were analyzed in the outlier removal program via SAS software.

This final stage of outlier determination was used to retain as much data as possible while not allowing outliers to adversely affect the overall result. Specifically, the generalized extreme Studentized deviate approach was applied to each sample that was based on five DBS (21). This method allows for the simultaneous detection of multiple outliers, in this case, two. Note that the position of up to two outliers can be found at either or both extremes of the sample. Once the outlier(s) were removed from the data set, a new CV was calculated with the remaining values. If the new CV < 15%, the 20% trimmed mean of the remaining values was accepted and reported. If no outliers were removed or the new CV ≥ 15%, the average of the 20% trimmed means from both assays was calculated and reported (Figure 2). These samples (very few, see results section) were not removed as we could not exclude that the observed values represent true variability.

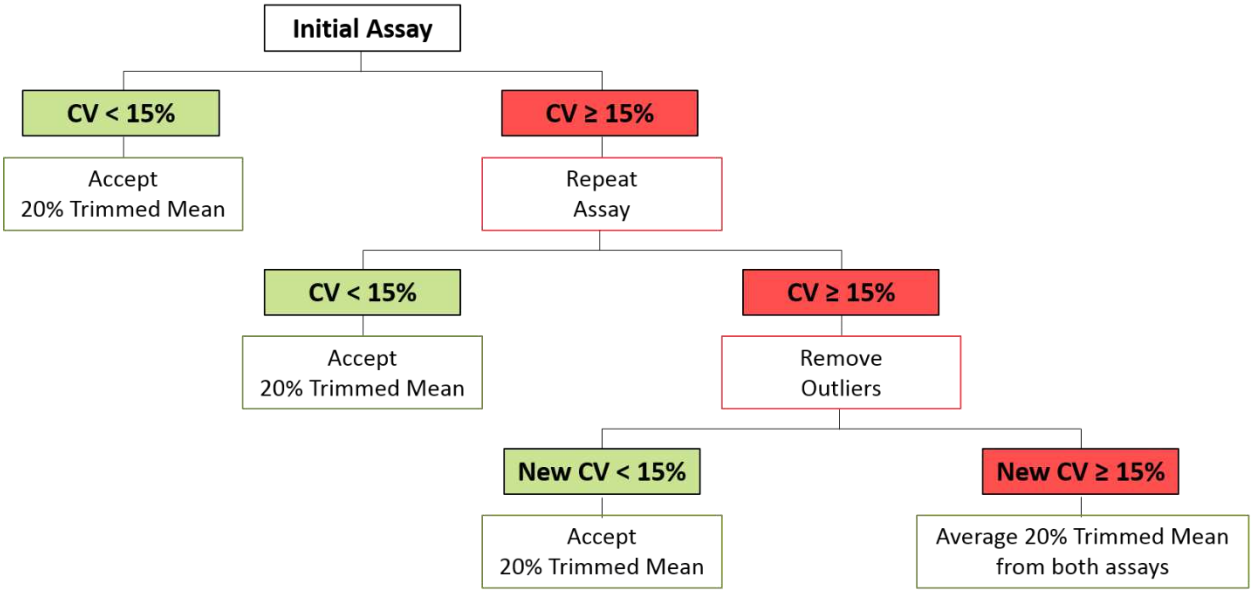

Figure 2. Result acceptance decision tree.

Correlation of Fragile X Protein Blood Level and Intellectual Function

In order to assess the potential clinical significance of FXP in blood as measured by our optimized assay, we examined the linear and non-linear relationship between peripheral FXP and intellectual functioning. For this, we examined a subset of participants with FXS (n=53) who completed the Abbreviated Battery of the Stanford-Binet, Fifth Edition (SB-5 (22)) as part of research evaluations (Table 2). SB-5 full-scale IQ standard scores were converted to deviation scores to provide a better estimate of intellectual ability in FXS participants (22, 23).

Table 2. Sub-set of Participants with FXS Included in IQ Correlation Analysis

	Male		**Female**		**Total**	
	FXS (n=28)	TDC (n=15)	FXS (n=24)	TDC (n=4)	FXS (n=52)	TDC (n=19)
Age	29.9 (8.8)	27.8 (8.3)	21.8 (8.6)*	17.3 (1.3)	26.2 (9.5)	25.6 (8.6)

Full-Scale IQ	46.5 (4.0)***	111.3 (13.6)	76.4 (20.0)	96.3 (9.6)	60.3 (20.4)***	108.1 (14.1)
Deviation IQ	32.2 (17.1)***	108.9 (13.0)	77.5 (18.1)	93.6 (8.9)	53.1 (28.7)***	105.7 (13.6)

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232

233 **Results**

234 **Immunoassay Optimization: Standard Curve and Lower Limit of Detection**

235 Unidentified components in whole blood can cause an overall decrease in signal intensity in
236 fluorescence-based immunoassays like the Luminex assay, termed the blood matrix effect (24, 25, 26).
237 To test if the standard curve conditions used in the Luminex FXP assay accounted for the blood matrix
238 effect, we used dried blood spot eluates from two fully methylated (FM) FM males as the dilution buffer
239 for the standard protein, GST-SR7. We compared standard curves with this "blood buffer" against the
240 standard curve made with the Luminex buffer previously used (15, 22, 23) and a standard curve made
241 with the elution buffer used for sample preparation (Figure 3). We observed that the blood components
242 globally decreased the median fluorescence intensity at each standard point compared with the
243 respective standard points prepared in Luminex buffer, suggesting that previously reported values may
244 have overestimated FXP in the blood. By contrast, the elution buffer closely mimicked the signal-
245 diminishing effect of the blood matrix. The patient blood curves showed variation in relative intensity,
246 most likely due to minute levels of endogenous FXP. Nevertheless, the elution buffer sufficiently
247 mimicked the signal-diminishing effects of the blood matrix while providing accurate representation of
248 sample preparation and was therefore used in this study.

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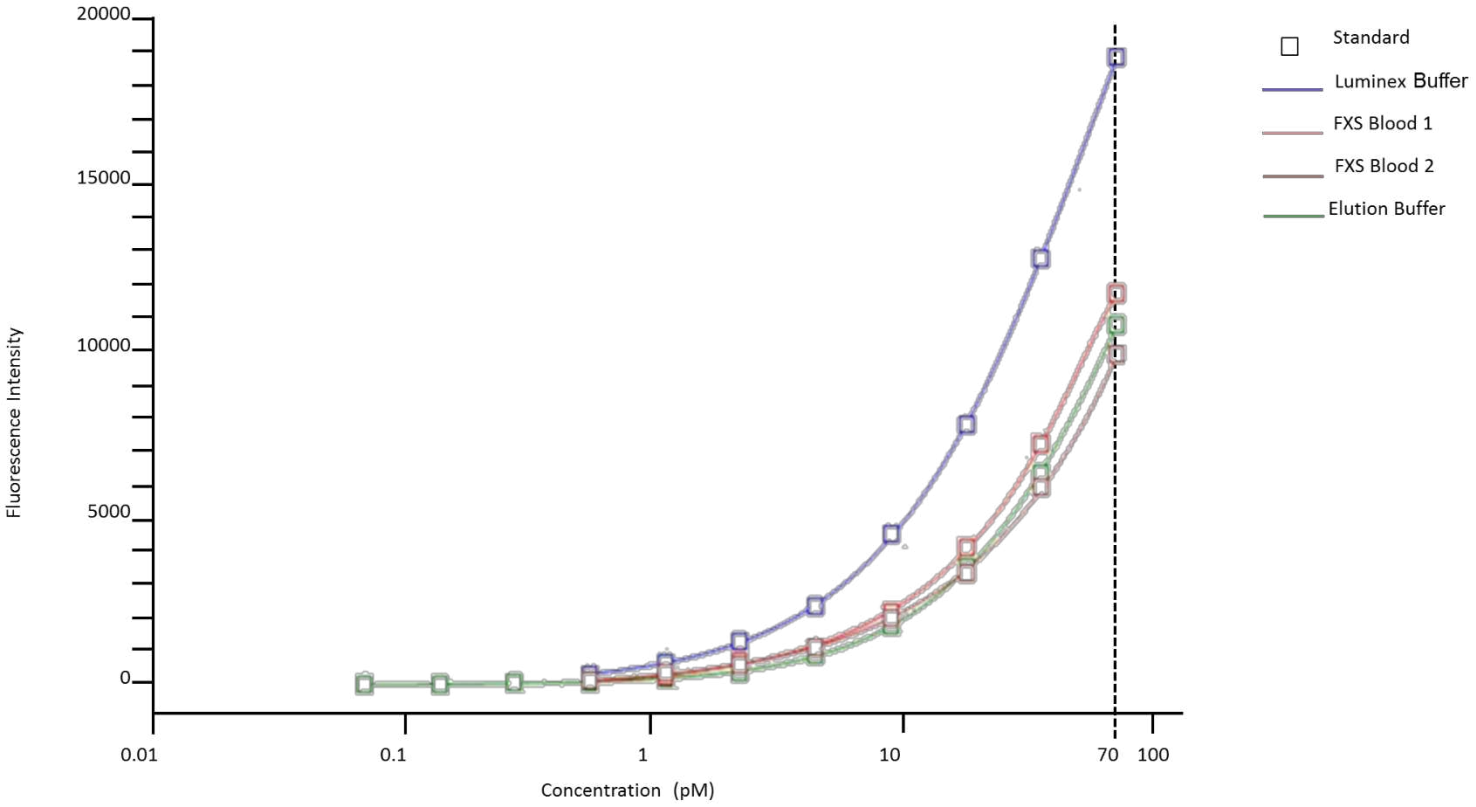


Figure 3. Elution buffer approximates blood matrix effect better than Luminex buffer. Luminex buffer (in blue) was previously used as the standard curve diluent. Due to components within the blood sample, demonstrated in the FXS blood curves (both red), fluorescent intensity is globally reduced. This blood matrix effect is mimicked by the elution buffer (in green), allowing the addition of more standard points to lower the detection limit to 0.07 pM.

We then evaluated the other components of the assay to negate any potential complications of interference or background due to the new buffer. Previous optimizations of ELISA-based methods (11, 27) utilized a checkerboard titration system to test multiple antibody conditions on the same test plate. Using this process, we compared different assay conditions to optimize magsphere preparation methods as well as compare two distinct detection antibodies, each at varying concentrations. The largest ratio of median fluorescence intensity between high and low standard points while maintaining a low background signal identified the optimized assay conditions. We determined that using a rabbit anti-human FXP (Abcam, ab17722) provided the highest signal-to-noise ratio with the optimized assay conditions (additional file 1).

Since the optimized assay conditions decreased the overall background of the assay, we added additional standard points to the low end of the standard curve which decreased the lower limit of detection of the assay. We consistently and reliably have decreased the lower limit of detection to 0.07 pM (Figure 3). This allows for a more accurate and quantifiable measure for patients with low levels of FXP which had been extrapolated, not quantified, using previous methods.

Analytical Validation of the Immunoassay

Intra-assay variability, or the variability between replicates, was measured and reported as an average %CV for each set of quintuplicates across all participant samples and plates. The intra-assay variability could not be calculated for 119 out of 527 (22.5%) of all quintuplicates because the values were outside of the limits of detection and therefore unquantifiable, which was expected since discernable FXP concentrations would not be expected for all FM FM males. The intra-assay variability was poor for 19% of the quintuplicates while good and acceptable for the remaining 60% of assays (47% of which had excellent variability). The overall average of the intra-assay variability (Table 3) was congruent with similar methods (11, 20,21) and our threshold for outlier removal.

Table 3. Performance statistics of the optimized FXP Assay.

Variability	**Sample Size**	**%CV ± SD**
Intra-Assay	553 (quintuplicates)	10.1 ± 7.9
Inter-Plate	41 (replications)	9.4 ± 10.2
Inter-Draw	26 (replications)	6.0 ± 4.1
Inter-Card	20 (replications)	2.8 ± 2.3

To determine the variability between plates (inter-plate variability), blood spots collected on the same DBS card from one patient were analyzed concurrently on two plates. The assays were prepared

independently of each other, using separate reagents and randomized analysis order. The inter-plate variability was determined by calculating the CV between the 20% trimmed means from both assay plates. The variability was poor for 15% of replications and good and acceptable for 70% of replications, and the remaining 15% of replications were below the lower limit of detection so therefore the %CV could not be calculated. The overall average inter-plate variability was congruent with the intra-assay variability (Table 3).

Since one K2EDTA collection tube contains enough blood to produce two DBS cards, we determined the inter-card variability, or the variability between both cards from the same blood draw. This value was determined by analyzing each card in quintuplicate on the same assay, prepared with the same conditions applied to each well. Some replications (25%) that were below the lower limit of detection and thus not quantifiable. This average inter-card variability was lower than the overall variability (Table 3).

Strategy for Outlier Removal

The outlier removal analysis was only necessary for 47 quintuplicate samples (out of 527 total sample runs, i.e. 8.9%)). Upon completion of the repeated assay, 33 of the samples had acceptable CVs. The remaining 14 repeated samples underwent outlier removal with 9 samples having successful removal of outliers. Only 4 (1 male and 1 female TDC, 2 fully methylated full mutation (FM FM) males with FXS) samples had a new CV ≥ 15% and thus have reported values of the average 20% trimmed means from both assays.

Assessing FXP values across diagnostic groups

With the optimized assay conditions, we quantified peripheral FXP in DBS eluate from a total of 187 individuals across the diagnostic categories. Values are reported as a concentration (pM) of FXP in the DBS extract.

We first evaluated the expression of FXP between the diagnostic categories. FXP levels are significantly reduced in individuals with FXS when compared with premutation carriers and TDCs respectively. There was no significant difference in protein concentration between premutation carriers and TDCs (Figure 4A). We then analyzed the effects of sex on FXP. There was no significant difference between sex within the premutation carrier category nor within the TDCs (data not shown). However, as expected, there was a significant reduction in FXP in males with FXS in comparison to females with FXS (Mann-Whitney U=20, n_1=70 n_2=33, $p < 0.0001$ two-tailed) (Figure 4B).

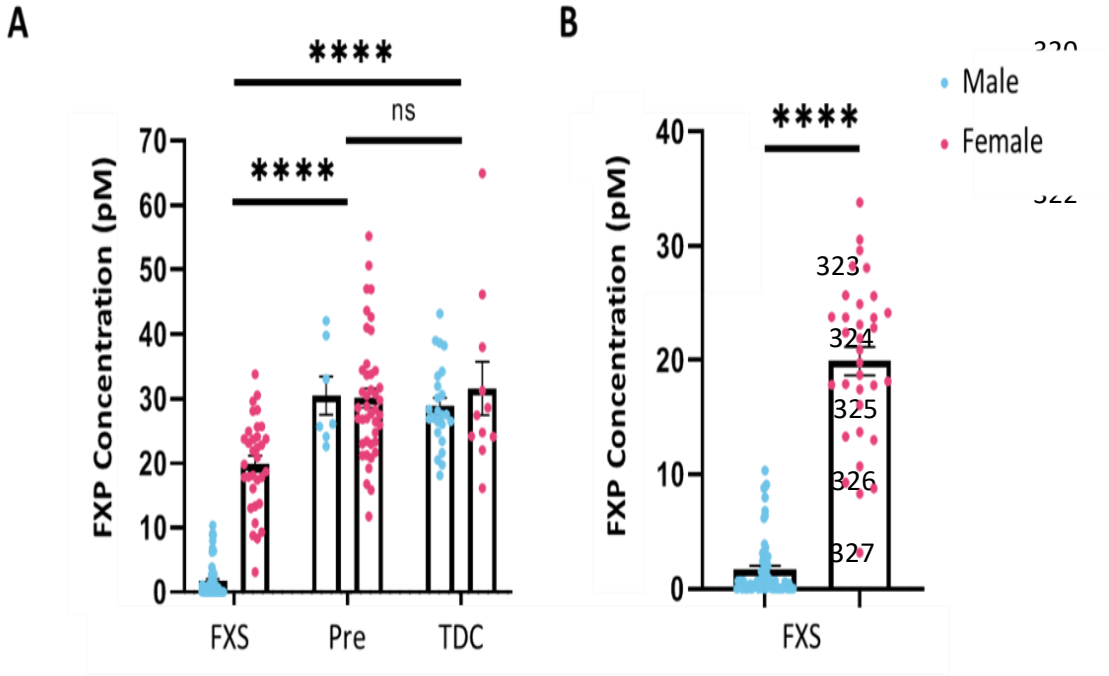
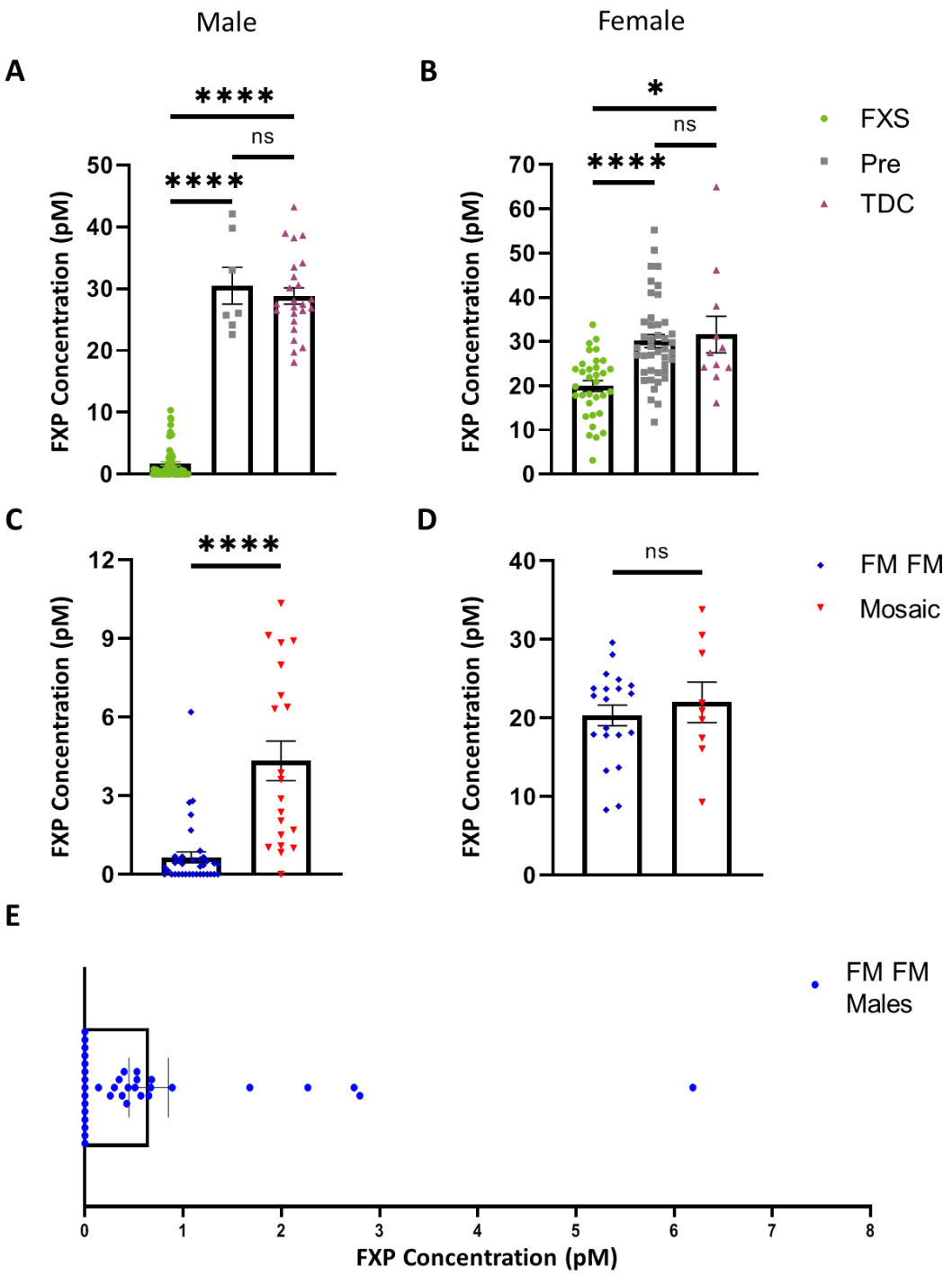


Figure 4. FXP concentration is reduced in FXS. (A) FXP concentration in all individuals across diagnostic categories from dried spot eluate. Graphically males and females are separated into individual bars; however, they were grouped as a diagnostic category when analyzed statistically. FXP concentration is significantly lower in the FXS diagnostic group than premutation carriers and TDC diagnostic groups (Kruskal-Wallis analysis with Dunn's multiple comparisons; H(3) = 107.2, , p(FXS vs Pre) < 0.0001, p(FXS vs Pre) < 0.0001). (B) FXP concentrations are significantly lower in males with FXS than females with FXS (Mann-Whitney U = 20, n_1 = 70 n_2 = 33, $p < 0.0001$ two-tailed). Mean reported with error bars representing SEM

Due to the nature of X-linked disorders and the fact that females are obligate mosaics, we then compared groups within sexes individually. Males with FXS had significantly lower FXP than their premutation carrier and TDC counterparts. There was no difference between premutation males and TDC males (Figure 5A). We observed the same trend in the females; females with FXS had significantly lower FXP than premutation carriers and typically developing controls, though there was no difference in FXP concentration between female premutation carriers and female typically developing controls (Figure 5B).



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347 **Figure 5**. FXP concentration is reduced in males and females with FXS. **(A)** Males with FXS have
348 significantly reduced FXP concentrations than premutation carriers ($p < 0.0001$) and typically developing
349 controls (Kruskal-Wallis analysis with Dunn's multiple comparisons; H(3) = 64.40 , p(FXS vs Pre) < 0.0001,
350 p(FXS vs Pre) < 0.0001). In our sample, there is no significant difference between FXP concentrations in
351 premutation carriers and typically developing controls (Kruskal-Wallis analysis with Dunn's multiple
352 comparisons; H(3) = 64.40 , p >0.9999). **(B)** Females with FXS had significantly lower FXP than
353 premutation carriers and typically developing controls (Kruskal-Wallis analysis with Dunn's multiple

comparisons; H(3) = 22.12 , *p(*FXS vs Pre*)* < 0.0001, *p(*FXS vs TDC*)* = 0.0101). There was no significant difference between female premutation carriers and TDCs (Kruskal-Wallis analysis with Dunn's multiple comparisons; H(3) = 22.12 , *p* >0.9999). Not that data shown in A and B are the same as in 4A. **(C)** FM FM males have significantly lower FXP concentration than males expressing mosaicism (Mann-Whitney U=68.5, n_1=36 n_2=20, *p* < 0.0001 two-tailed). **(D)** There is no significant difference between FM FM females and females with methylation mosaicism (Mann-Whitney U=84, n_1=20 n_2=9, *p* = 0.7992 two-tailed). **(E)** Higher resolution of data for FM FM males illustrates that they express varying levels of FXP, ranging from undetectable to over 6. Mean reported with error bars representing SEM

Next, we compared males that express the fully methylated (FM) FM to their mosaic male counterparts; here, mosaicism is a grouped category where individuals with either repeat or methylation mosaicism were analyzed as the mosaic group. As expected, FM FM males have significantly lower FXP than mosaic males. Interestingly, we found that there are some FM FM males that consistently express low amounts of FXP, but still less than the average mosaic male FXP level (Figure 5C). Since females are obligate mosaics due to their compensatory X chromosome, we defined mosaicism for females based on methylation status alone. Using this definition, we differentiated between FM FM females and mosaic females that express both full mutation and premutation bands after southern blot analysis. There was no significant difference between FM FM females and methylation mosaic females (Figure 5D).

Validation of Assay Reproducibility

We evaluated within subject test-retest reproducibility of blood FXP in two cohorts, a group of 12 adults with full mutation FXS with FXP levels measured every two weeks totaling seven FXP samples and in 25 persons sampled less frequently (a minimum of two FXP measurements per patient; duration between assays 0.5 to 30 months). In both the short-term (Figure 6A) and longer-term within subjects repeated FXP testing (Figure 6B), the FXP results showed excellent intra-individual stability (ICC values were 98.8 and 97.6, respectively). The average inter-draw variability was lower than the overall assay variability (Table 3).

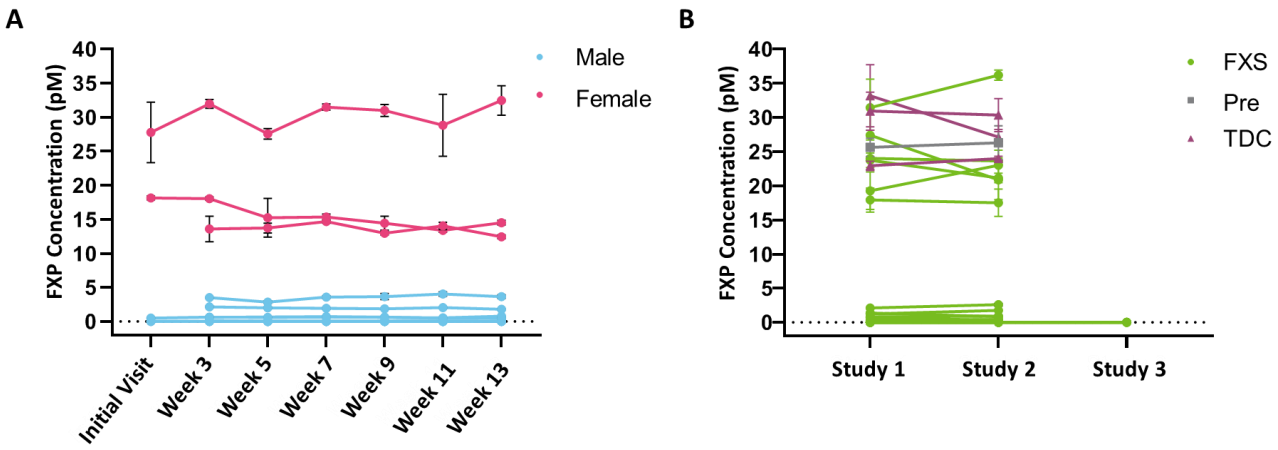

Figure 6. FXP concentrations are consistent over time. (A) Repeated bi-weekly FXP levels in 12 adults with FM FXS (9 males; 3 females) showed consistency over 13 weeks (ICC value 98.8) (B) Over longer intervals between FXP testing (mean 11 months; range 0.5- 30 months), within subject FXP levels showed consistency (ICC value 97.6) in 25 persons (18 males, 7 females; age range 0.5-49.8 years) with FXS, premutation, or no FX-related finding. Note that study 3 included multiple FM FM males with undetectable FXP levels.

Correlation of FXP levels with intellectual function

In both males (r_{LIN}=.38 p=.04) and females (r_{LIN}=.53, p=.01) with full mutation FXS, higher FXP concentrations were associated with higher Deviation IQ scores. However, when removing six male participants who had either size or methylation mosaicism, this relationship was no longer significant for males with FXS (r=.15, p=.28). We next examined males with mosaicism and females together, with the thought that because mosaicism confers some production of FXP, the underlying mechanism supporting this relationship with intellectual functioning may be more similar to females with FXS than FM-FM. Deviation IQ and FXP remained significantly related (r_{LIN}=.73 p<.001).

Previous studies have indicated a non-linear model best describes the relationship between FXP and IQ in FXS (ref). Here, we conducted multiple non-linear models to determine which non-linear function best fit our data separately for males (Figure 7a) and females (Figure 7b) with FXS. Among males with FXS, a linear model remained the best fit. In contrast, among females with FXS, logarithmic (r_{LOG}=.56, r^2=.31, p=.005) and inverse (r_{INV}=.56, r^2=.31, p=.005) functions also fit the data.

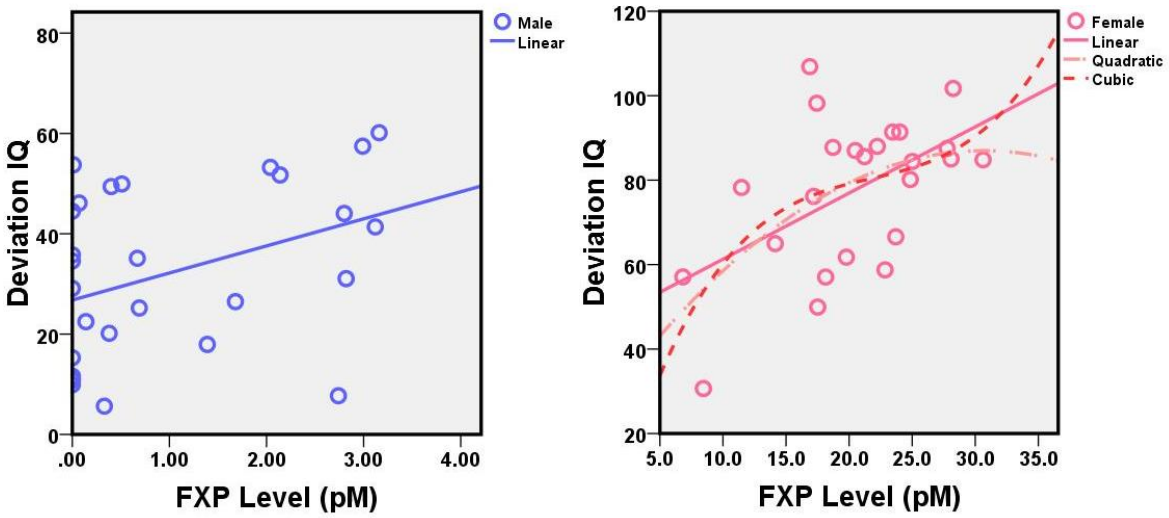

Figure 7. Linear and non-linear correlations between FXP concentrations and Deviation IQ scores for males (A) and females (B) with FXS

Next, due to the discrepancy in FXP concentration levels between males and females with FXS, we normalized the data with log10 function in order to further assess relationship to IQ with the larger FXS sample. We found significant relationships with linear ($r=.69$, $r^2=.46$, $p<.001$), quadratic ($r=.83$, $r^2=.68$, $p<.001$), and cubic ($r=.83$, $r^2=.69$, $p<.001$) functions.

Discussion

Given FXS is defined at the core as a protein deficiency disorder, study of FXP is the most directly linked potential protein marker of disease in this field. Thus, the development of a continuous, reliable, and clinically relevant biological marker of fragile X pathophysiology is of critical importance to translational treatment development in this field. Such a marker would have potential clinical utility to predict patient subgroups that may best respond to treatment while also potentially serving as a future moveable biological target of treatment itself. Our current work demonstrates that FXP as measured in peripheral blood by our optimized assay holds promise as a reliable and clinically relevant biological marker in the FXS field.

Previous methods of quantifying FXP have had low signal to noise ratios that increase at the lower limit of quantification and therefore mask potential low level protein expression (19). Although these previous methods have shown initial clinical relevance by documenting relationships with general cognitive ability, due to the restricted range of FXP expression captured by these methods, the full scope of its clinical utility is limited (19). In the current study, using several key assay optimization techniques, including standard curve and reagent optimization, we have developed a reliable and reproducible means to quantify FXP in blood using blood spot cards. Using extensive test-retest strategies we have validated reproducibility of this assay across a wide range of FXP values. In conjunction with lowering the limit of detection to 0.07 pM and our optimizations resulting in increased sensitivity, we identified a new distinct sub-population of clinically defined full mutation fully methylated (FM FM) males expressing trace or very low levels, but not absent, FXP. This is the first time, to the best of our knowledge, that an FXP assay has reliably and reproducibly documented very low levels of FXP in FM FM in the context of also identifying subjects with zero FXP. Yet, among FM FM males alone (i.e., excluding males with mosaicism) relationship between FXP and IQ was no longer significant. This suggests that FXP below a specific threshold may no longer be related to intellectual functioning. Replication and further exploration of this result is needed as is a more comprehensive examination of clinical implications of trace production.

Our study results must be taken in the context of several weaknesses. First, our sample size of premutation fragile X carriers and control participants was limited. Given this, a thorough interpretation of premutation FXP levels and their clinical relevance will require future work to enroll larger samples of male and female PMCs to better evaluate PMC FXP expression profiles. Though we can differentiate mean FXP differences between persons with FXS and control subjects in this small sample, increasing our control sample numbers will be important in the future to better define what would be considered a "normal FXP range" in humans. Previous studies used "housekeeping genes", genomic DNA or white

blood cell counts to normalize FXP values in peripheral blood (19). Given the widespread effects on protein synthesis by loss of FXP and limited knowledge about the differential expression of FXP across blood cells, here, we chose not to include a normalization step. Future studies are needed to identify the optimal factors for normalization that are not affected by FXS diagnosis. Additionally, we lack at this time data assessing peripheral FXP expression across development in all patient groups evaluated. It will be important to evaluate for potential developmental shifts in FXP expression across developmental windows. Such information will be imperative to interpreting FXP findings and predicting their clinical impact while also enhancing our understanding of FXS pathology thus aiding potential protein-focused therapeutics development in the future.

In addition, our clinical data presented in this manuscript is limited to IQ alone. Comprehensive, multimodal phenotyping of humans with defined FXP blood levels is needed to better understand the potential FXP-brain-behavior relations that may exist in FXS, PMCs, and in the control group populations. Although we have demonstrated potential associations between FXP expression and general cognitive function in FXS, we need to use more quantitative and direct evaluations of brain function such as high density electrophysiology, neuroimaging, and additional performance based measures to understand potential relationships between FXP expression and human phenotypes. In particular, it will be imperative to increase our subject sample size in the context of deep phenotyping to determine the clinical relevance, if any, of trace versus zero FXP expression in FM FM males with FXS. Given our FXP analysis is a peripheral tissue assay, clear challenges exist regarding whether a blood finding correlates with true brain FXP variance in humans. Comprehensive neurophysiologic, behavioral, and cognitive phenotyping will play a role in addressing this underlying question as will potential future post-mortem study to evaluate FXP across tissues including brain FXP expression analysis. We remain hopeful that enhanced quantification of brain neurophysiology will in the near term enhance our ability to evaluate the impact of FXP expression as measured in blood to brain activity and function.

Last, to date, we have not evaluated for the potential molecular reasons why we are detecting trace FXP expression in certain males with FM FM FXS. Given the large number of methylation sites on the FMR1 gene, we hypothesize that regular Southern Blot and PCR testing potentially lacks the sensitivity to detect small deviations from true full methylation which could result in some transcription of the FMR1 gene and resultant FXP production. Future in depth molecular study is warranted to further understand human FMR1 methylation patterns while also evaluating FXP expression across patient groups in the context of RNA transcript composition and expression. Such future work may be applicable beyond FXS to understand mechanisms of breakthrough protein expression in genes thought to be completely silenced.

Conclusions

FXP can be reliably and reproducibly quantified via peripheral whole blood in humans. Variance in FXP was expected and noted between males and females with FXS as well as between groups of persons with FXS versus PMC and non-FX impacted control groups. We have demonstrated the ability to reliably detect very low or trace levels of FXP in blood. In doing so, we have discovered a cohort of males with FXS clinically characterized at having a FXS full mutation fully methylated FMR1 allele who are expressing FXP in their blood. Consistent with prior work in this field, we observed clinical associations between peripheral FXP expression and general cognitive functioning indicated. Future work is necessary to understand both the clinical relevance and molecular mechanisms of trace FXP expression in a subpopulation of males with FXS.

Additional Files

Additional File 1. Antibody optimization via the checkerboard titration method. Capture antibody buffer exchange was optimized due to concerns regarding the efficiency of the bead-antibody coupling reaction.

Detection Antibody	Capture Antibody Buffer Exchange	Detection Antibody Concentration											
		1:625			1:1250			1:2500			1:3125		
		70 pM	0.55 pM	0 pM	70 pM	0.55 pM	0 pM	70 pM	0.55 pM	0 pM	70 pM	0.55 pM	0 pM
R477	-	12570	409	293	11038	280	124	10463	206	69	7746	135	45
		12023	490	213	11674	331	128	9843	185	60	9181	172	47
	+	12705	414	195	11698	320	115	9978	215	57	9490	169	83
		13805	418	164	13924	303	139	12047	207	55	11072	160	46
		Detection Antibody Concentration											
		1:100			1:250			1:500			1:1000		
		70 pM	0.55 pM	0 pM	70 pM	0.55 pM	0 pM	70 pM	0.55 pM	0 pM	70 pM	0.55 pM	0 pM
ab17722	-	11165	196	66	10263	172	50	9599	170	46	10142	155	40
		11143	219	63	10905	180	53	9396	168	46	9483	154	39
	+	13807	230	59	13343	200	46	11804	186	38	11798	177	39
		13324	222	56	12453	208	45	10782	195	44	11600	162	37

List of Abbreviations

CV	coefficient of variation
DBS	dried blood spot
FM FM	full methylated, full mutation
FMRP	fragile X mental retardation protein
FXP	fragile X protein
FXS	fragile X syndrome
ICC	intra-class correlation coefficient
ID	intellectual disability
IQ	intelligence quotient
NS	non-significant
pM	picomolar
Pre or PMC	premutation carrier
SEM	standard error of the mean

SB-5 Stanford Binet 5th Edition

TDC typically developing control

Declarations

Ethics approval and consent to participate

Manuscripts reporting studies involving human participants, human data or human tissue must:

All human experiments described in this publication were approved by the Cincinnati Children's Hospital Medical Center Institutional Review Board (IRB # 2013-7327). All human subjects when able provided informed consent for all study procedures and all subjects under guardianship (minors or adults) had their guardian consent for subject participation with assent, when possible, obtain from the subject him or herself.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors have no financial interests specifically related to the content of this manuscript. CAE is a current consultant to Confluence, Stalicla, Impel, Forge, and Scioto Bioscience. CAE is the inventor on patents related to the treatment of autism and/or fragile X syndrome.

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Authors' contributions

AEB and CG participated in experiment design and execution and wrote and edited the manuscript. LMS participated in experiment design and wrote and edited the manuscript. KDC edited the manuscript and participated in data interpretation and design. PSH provided the statistical analyses in the manuscript and edited and approved the manuscript. RM, TA and GL participated in early elements of experimental design and edited and approved the manuscript. CAE conceived the overall experiments, participated in experimental design, data interpretation, and wrote and edited the manuscripts. All authors edited and approved the final manuscript.

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