

# Comparison Between the Polymerase Chain Reaction-Based Screening and the Southern Blot Methods for Identification of Fragile X Syndrome

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The fragile X syndrome (FXS), the most common cause of hereditary mental retardation, is caused by expansions of CGG repeats in the *FMR1* gene. The gold-standard method to diagnose FXS is the Southern blot (SB). Because SB is laborious and costly, some adaptations in the polymerase chain reaction (PCR) method have been utilized for FXS screening. A previous PCR-based screening method for FXS identification utilizing small amounts of DNA was reported as simple and efficient. The aim of this study was to reproduce the mentioned PCR-based screening method for identification of expanded alleles of the *FMR1* gene in Brazilian individuals and to investigate the efficiency of this method in comparison with SB. Utilizing the enzyme Expand Long Template PCR System, 78 individuals were investigated by that PCR-based screening method for FXS identification. Conclusive results were obtained for 75 samples. Considering all the allelic forms of FXS (normal [NL], premutation [PM], and full-mutation [FM]), the comparison of the PCR-based screening method with SB demonstrated 100% of accuracy, sensitivity, and specificity. However, when the PM and the FM were analyzed separately from each other, but together with the NL allele, the accuracy, sensitivity, and specificity decreased (to 42.9%–97.4%). We concluded that the PCR-based screening method was reproducible and capable of identifying all different FXS alleles, but because the differentiation between the PM and the FM alleles was not accurate, SB is still the gold-standard method for the molecular diagnosis of FXS.

## Introduction

**T**HE FRAGILE X SYNDROME (FXS) is the most common inherited cause of intellectual disabilities and the most common single genetic cause of autism (Hagerman *et al.*, 2008). This is occurred by the instability and expansion of CGG repeats in the 5'-untranslated region (5'-UTR) of exon 1 of the *FMR1* gene together with the occurrence of hypermethylation (Verkerk *et al.*, 1991). The CGG-repeat element is polymorphic, varying in general from 5 to 44 repeats in the normal (NL) range, from 45 to 54 repeats in the gray zone, from 55 to 200 repeats in the premutation (PM) range, and over 200 CGG repeats in the full-mutation (FM) range (Crawford *et al.*, 2001; Hagerman and Hagerman, 2004). Expansion to the FM occurs when PM alleles are transmitted maternally to offspring (Coffee *et al.*, 2009).

Many techniques for identification of FXS have been reported in the literature, among them, molecular tests (Rous-

seau *et al.*, 1991; Haddad *et al.*, 1996; Hamdan *et al.*, 1997; Panagopoulos *et al.*, 1999; Saluto *et al.*, 2005; Dahl *et al.*, 2007; Rosales-Reynoso *et al.*, 2007; Dodds *et al.*, 2009; Chen *et al.*, 2010; Filipovic-Sadic *et al.*, 2010). Two main diagnostic techniques have been developed for the DNA-based diagnosis of FXS: Southern blot (SB) and polymerase chain reaction (PCR) (Sofocleous *et al.*, 2009). SB is considered the gold-standard method for diagnosis of FXS, in spite of it is laborious and costly.

The use of PCR for FXS screening suffers some methodological difficulties, such as amplification of large-sequence rich-oin C and G nucleotides, which led several researchers to propose modifications in this method (Haddad *et al.*, 1996; Hamdan *et al.*, 1997; Panagopoulos *et al.*, 1999; Saluto *et al.*, 2005; Dahl *et al.*, 2007; Rosales-Reynoso *et al.*, 2007). Tassone *et al.* (2008) reported a PCR-based screening method for FXS that seems to be revolutionary, because only PCR would solve most of the suspicious cases of the syndrome (PM or FM allele carriers in both genders, including mosaics).

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Considering the potential usefulness of the PCR-based screening method described by Tassone *et al.* (2008) for rapid identification of expanded alleles of the *FMR1* gene, the aim of this study was utilize it in Brazilian individuals and verify its efficiency through analysis of accuracy, sensitivity, and specificity in comparison with SB.

## Materials and Methods

### Samples

DNA samples from 78 individuals, being 40 males and 38 females, some of them suspicious of FXS and also relatives, mainly their mothers (6 in total) were used in the current study. These 62 samples were provided by Dr. Débora Aparecida Rodrigues from the School of Medical Sciences and Health (FCMS/PUC-SP) and by Dr. Angela Maria Vianna-Morgante from the Biosciences Institute of São Paulo—USP. The other 16 samples were collected from patients referred by Dr. Valter Curi Rodrigues, from the University Center of Araraquara (UNIARA). This study was approved by the Committee for Ethics Affairs of UNESP—São Paulo State University, Faculty of Pharmaceutical Sciences (Protocol number 24/2008).

### Molecular analysis

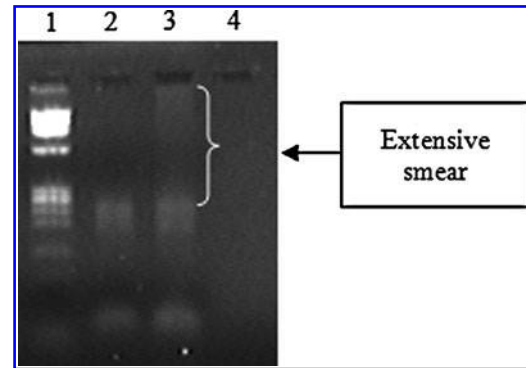
Drops of peripheral blood from each patient were collected with an FTA card (Whatman, Inc.). A disk 1.5 mm in diameter was removed by punch from each dried blood spot, and the proceedings of card purification were made according to Tassone *et al.* (2008). Each purified sample was numbered by another participant researcher, so that the study could be conducted in a blind way.

Afterward, the PCR-based screening method for FXS identification described by Tassone *et al.* (2008) was developed. The first and second PCR reactions were performed using the master mix containing 0.33  $\mu$ M of each c and f primers (Fu *et al.*, 1991), 500  $\mu$ M of dNTPs, 2.2 M of betaine (USB), and one disk of 1.5-mm FTA card. The PCR cycling conditions included initial denaturation at 98°C for 10 min; 68°C for 2 min (hot start, with the addition of 0.5 U of Expand Long Template PCR System [Roche Diagnostics] enzyme with buffer 2 [1 $\times$ ]), followed by 10 cycles at 97°C for 35 s, 64°C for 35 s, and 68°C for 4 min; 25 cycles at 97°C for 35 s, 64°C for 35 s, and 68°C for 4 min, plus a 20-s increment for each cycle; and a final extension at 68°C for 10 min.

Because some information such as enzyme concentration and hot start details was not observed in the article published by Tassone *et al.* (2008), the PCR method had to be standardized by our group. Here, we utilized 0.5 U of the Expand Long Template PCR System (Roche Diagnostics) enzyme and the hot start, as was detailed above.

The PCR products were electrophoresed at 6 V/cm for 60 min on a 2% TBE 1 $\times$  agarose gel containing ethidium bromide. The gels were visualized on a transilluminator and photographed in the GDS 8000 System (UVP). The expected constant region of the PCR product presents an amplicon of 221 bp (i.e., excluding the expanded CGG repeats).

Samples that did not produce a band in the first PCR were subjected to a secondary PCR screen with the c primer and the CGG-chimeric primer described by Tassone *et al.* (2008) (Fig. 1). In that second PCR, were used the same reagents and cycling conditions mentioned above.



**FIG. 1.** Detection of expanded alleles using CGG-targeting polymerase chain reaction (PCR) primer; 2% agarose gel. Lane 1: DNA size marker PhiX 174 *Hae*III (New England). Lane 2: Sample of individual with normal (NL) allele for the FXS. Lane 3: Sample of individual with fragile X syndrome (FXS) full-mutation (FM) allele (an extensive smear is displayed in the presence of the allele). Lane 4: Control negative.

In addition, as suggested by Tassone *et al.* (2008), to eliminate the hot start procedure (necessary when utilizing the Expand Long Template PCR System enzyme), we also made efforts to standardize the utilization of the FastStart *Taq* DNA Polymerase (Roche Diagnostic) enzyme. However, important details that are necessary for the proper use of this polymerase were absent in the aforementioned report, such as (1) which buffer (and its concentration) had to be used; (2) how many enzyme units should be required; and (3) which cycling conditions should be utilized. Therefore, the lack of all this information offered difficulties for the success in reproducing the PCR-based screening method for FXS described by Tassone *et al.* (2008), and then we tried to adapt it. After some attempts, the best conditions reached by using the FastStart *Taq* DNA Polymerase enzyme for the first and second PCRs were a master mix containing 0.33  $\mu$ M of each c and f primers (Fu *et al.*, 1991), 0.5 U of the enzyme with buffer 2 (1 $\times$ ) without  $MgCl_2$ , 2.25 mM of  $MgCl_2$ , 500  $\mu$ M of dNTPs, 2.2 M of betaine (USB), and a disk of 1.5-mm FTA card. The PCR cycling conditions were as follows: initial denaturation at 98°C for 10 min, followed by 10 cycles at 97°C for 35 s, 64°C for 35 s, and 72°C for 4 min; 25 cycles at 97°C for 35 s, 64°C for 35 s, 72°C for 4 min plus a 20-s increment for each cycle; and a final extension at 72°C for 10 min. PCR products were electrophoresed at 6 V/cm for 60 min on a 2% TBE 1 $\times$  agarose gel containing ethidium bromide.

Concomitantly, to analyze the efficiency of the PCR-based screening method for FXS identification, all the samples were also submitted to FXS diagnosis by the SB method, which were performed by an outsourcing laboratory (using the probe StB12.3 and enzymatic digestion with *Eco*RI and *Eag*I) (Mingroni-Netto *et al.*, 1996).

After obtaining the results of the PCR-based screening method for FXS, they were compared, for each patient, with the SB results (gold-standard method). Contingency tables (2 $\times$ 2) were generated to assess the efficiency of identification of the FXS alleles by the PCR method described by Tassone *et al.* (2008). Accuracy, specificity, and sensibility were calculated utilizing the BioEstat v. 5.0 (Ayres *et al.*, 2007).

## Results

Samples selected to amplify in the in the secondary PCR gave products as showed in the Figure 1. An extensive smear was produced with the chimeric primer when an expanded allele was present (lane 3), but no smear was visible in the presence of the NL allele (lane 2). These results demonstrate that we were able to reproduce the PCR-based screening method for FXS identification from Tassone *et al.* (2008) when utilizing the Expand Long Template PCR System enzyme.

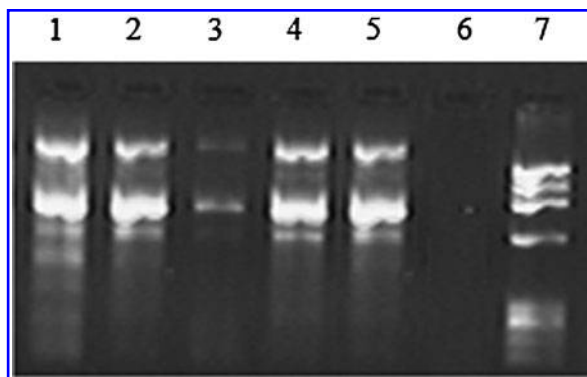
In regard to the standardization of the Tassone *et al.* (2008) method using the FastStart *Taq* DNA Polymerase, we obtained success in amplifying the first PCR (data not shown), but for the second PCR, in spite of numerous attempts, only inconclusive results were achieved (Fig. 2).

Consequently, we decided to adopt the Tassone *et al.* (2008) PCR-based screening method for FXS identification with the Expand Long Template PCR System enzyme. A total of 78 samples from female and male subjects were analyzed. Samples from NL and PM females gave results consistent with that previous report for the first and second PCR (Fig. 3). From the 78 individuals investigated, conclusive results were obtained for 75 samples (96.2%).

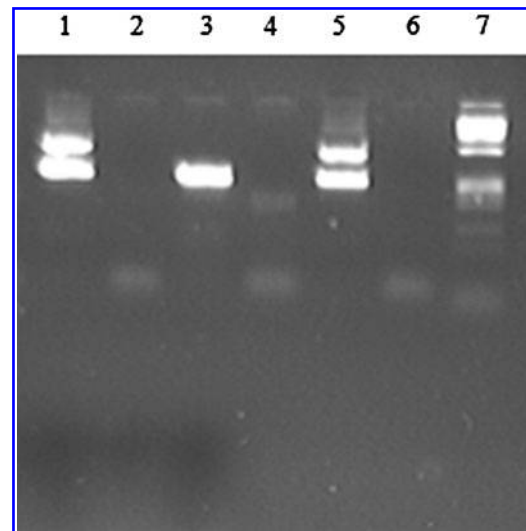
When the results of FXS diagnosed by the SB (gold standard) method were supplied by the outsourcing laboratory, they could be confronted with those results achieved by the PCR-based screening method for FXS (Table 1). Considering the total of 75 conclusive results by the PCR-based screening method for FXS, only five (6.7%) of them did not match with those provided by SB (Table 1). The accuracy, sensitivity, and specificity were evaluated considering the results of the FXS alleles provided by PCR in contrast with those obtained by SB. That analysis of the efficiency of the Tassone *et al.* (2008) method for identifying the FXS alleles was performed in three different forms considering (1) all the 75 conclusive results by PCR (Fig. 4A); (2) the presence of NL and PM alleles by PCR (34 samples) (Fig. 4B); and (3) the presence of FM and NL alleles by PCR (44 samples) (Fig. 4C).

## Discussion

In the present study, we assessed the accuracy, sensitivity, and specificity of the PCR-based screening method for FXS proposed by Tassone *et al.* (2008) in comparison with SB.



**FIG. 2.** A 2% agarose gel of second PCR products using the FastStart *Taq* DNA Polymerase. Lanes 1 and 2: Male subjects. Lanes 3 and 4: Female subjects, Lane 5: Female subject with NL alleles for FXS. Lane 6: Negative control. Lane 7: Molecular weight marker Phi X 174 *Hae*III (New England).



**FIG. 3.** A 2% agarose gel. Lanes 1, 3, and 5: Results of the first PCR (using the Expand Long Template PCR System). Lanes 2, 4, and 6: Results of the second PCR. Lanes 1, 2, 5, and 6: Female subjects carrying premutation (PM) alleles (in lane 1 and 5, PCR products confirmed the heterozygosity: NL and PM alleles; in lanes 2 and 6, PCR products do not appear, and this confirm the absence of expanded allele). Lanes 3 and 4: Female subject carrying NL allele (in lane 3, a single PCR product indicates homozygous for the NL allele; in lane 4, small PCR products are produced with the chimeric primer. This result confirmed the presence of only the NL allele). Lane 7: Molecular weight marker Phi X 174 *Hae*III (New England).

To eliminate the hot start step, Tassone *et al.* (2008) utilized the FastStart *Taq* DNA polymerase enzyme, but in spite of many attempts, we were not able to reproduce the Tassone *et al.* (2008) secondary PCR, which is why we decided not to use this enzyme. Another study conducted by the Tassone group also used the FastStart *Taq* DNA polymerase, which demonstrated satisfactory results (Fernandez-Carvajal *et al.*, 2009). Nevertheless, insufficient methodological details regarding the use of this enzyme were described. Although the authors stated that this method of PCR could detect all forms of FXS alleles, they continued looking for other molecular technologies to reach that objective. More recently, two different PCR methodologies were reported utilizing fluorescent primers and G-C-rich PCR reagents that were resolved by both capillary electrophoresis and agarose gel electrophoresis (Chen *et al.*, 2010; Filipovic-Sadic *et al.*, 2010).

Utilizing the Expand Long enzyme, we obtained 75 conclusive results from 78 individuals investigated. The three inconclusive results (3.8%) occurred because there was no amplification in the second PCR. Therefore, they were excluded from this study.

Among the 75 conclusive results obtained by the PCR-based screening method, five (6.7%) were not coincident with the SB diagnosis (Table 1). From those five samples, four samples were diagnosed as FM, because in the first PCR, there was no amplification of an allele in the range of the PM allele, and in the second PCR, an extensive smear was observed. Tassone *et al.* (2008) mentioned that this extensive smear indicates the presence of an FM allele. However, the SB analysis

TABLE 1. RESULTS OF BOTH POLYMERASE CHAIN REACTION-BASED SCREENING AND SOUTHERN BLOT METHODS FOR FRAGILE X SYNDROME IDENTIFICATION

Number of individuals (%)	Gender	PCR-based screening	Southern blot
01 (1.3)	Male	NL (1st and 2nd PCRs)	NL
02 (2.5)	Female	NL (1st and 2nd PCRs)	NL
10 (12.8)	Male	PM (1st PCR)	PM
02 (2.5)	Male	FM (1st and 2nd PCRs)	PM
20 (25.7)	Female	PM (1st PCR)	PM
02 (2.5)	Female	FM (1st and 2nd PCRs)	PM
26 (33.4)	Male	FM (1st and 2nd PCRs)	FM
11 (14.1)	Female	FM (1st and 2nd PCRs)	FM
01 (1.3)	Male	PM (1st and 2nd PCRs)	PM and FM (mosaicism)
03 (3.9)	Female	Inconclusive (1st and 2nd PCRs)	FM
Total 78 (100)			

PCR, polymerase chain reaction; NL, normal alleles; PM, premutation alleles; FM, full-mutation alleles.

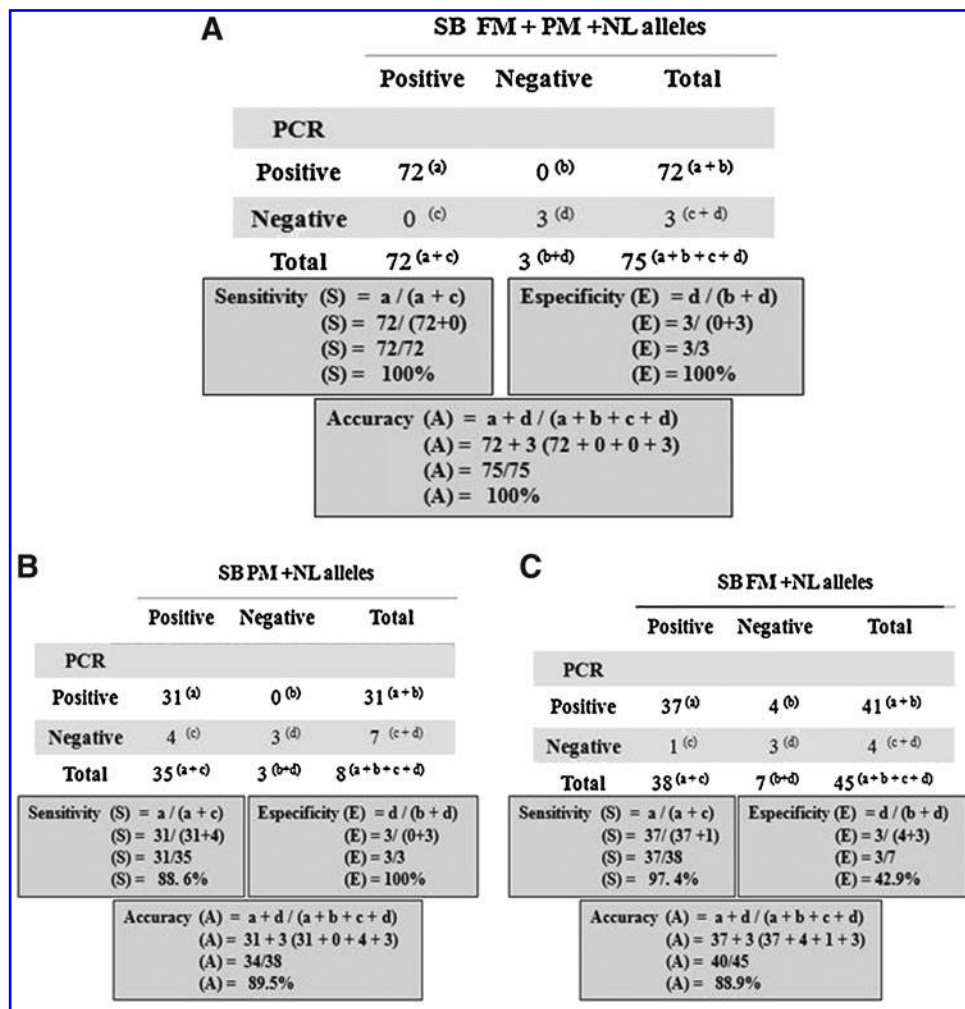


FIG. 4. Results of accuracy, sensitivity, and specificity. (A)—a: subjects carrying any FXS-expanded alleles detected by Southern blot (SB) that were also detected by the PCR (true positives); b: subjects that not carry FXS-expanded alleles detected by SB, but that were detected by the PCR (false-positive); c: subjects carrying any FXS-expanded allele detected by SB, which by the PCR they were not detected (false negative); d: subjects that not carry FXS-expanded alleles detected by SB, which were concordant by PCR (true negative); (B)—a: subjects that carry PM allele for FXS diagnosed by SB, and the PCR also diagnosed the PM allele (true positives); b: subjects that not carry PM allele for FXS by SB, but that PCR diagnosed PM allele (false positive); c: subjects that carry PM allele for FXS by SB and that PCR identify FM allele wrong (false-negative); d: subjects that not carry PM allele for FXS by SB, and that PCR not diagnosed none expanded allele (true negative); (C)—a: subjects that carry FM allele for FXS by SB and that PCR diagnosed this FM allele (true positive); b: subjects that not carry FM allele for FXS by SB, but that PCR method diagnosed FM allele (false positive); c: subject that carry FM allele for FXS by SB and that PCR method did not diagnose (false-negative); therefore, by PCR, this subject was identified only carrying of PM allele, without identifying the mosaicism; d: subjects that did not carry FM allele for FXS and that PCR did not diagnose none FM alleles (false negative).

detected PM alleles in these four samples (Fig. 4B-c). According to Tassone *et al.* (2008), the PCR-based screening method for FXS identification is capable to detect in the first PCR the alleles in the upper range of PM (~200 CGG repeats); nevertheless, even under conditions where the first PCR fail to detect a PM allele, the presence of such allele would be identified with the secondary PCR by the presence of an extensive smear. In those cases, the authors advised that SB should be used to definitively genotype the alleles. Therefore, it is understood that whenever there is the necessity of using the secondary PCR, and if the obtained results were an extensive smear, it is not possible to accurately distinguish between the PM or FM alleles (Tassone *et al.*, 2008). One can hypothesize that the reason for using the PCR methodology demonstrated by Filipovic-Sadic *et al.* (2010) and Chen *et al.* (2010) would be to find a method capable to identifying the number of GCC repeats that are expanded, generating all the forms of FXS alleles.

Regarding the fifth sample investigated by a PCR-based screening method that was not coincident with SB, the first PCR was able to identify a PM allele. Nevertheless, by SB, the sample was genotyped as mosaic for the PM and FM alleles (Fig. 4C-c). Based on this result, we decided to perform a second PCR, and the presence of an expanded allele was confirmed by an extensive smear.

Because it is important to correctly diagnose FXS in individuals, SB is considered the gold-standard method and has been utilized since 1991 (Rousseau *et al.*, 1991). However, as SB is a laborious and costly technique, numerous PCR-based procedures have been developed for rapid FXS screening (Haddad *et al.*, 1996; Hamdan *et al.*, 1997; Panagopoulos *et al.*, 1999; Saluto *et al.*, 2005; Dahl *et al.*, 2007; Tassone *et al.*, 2008). The screening methodological approaches should satisfy the principal requirements: to be rapid and cost effective (Fernandez-Carvajal *et al.*, 2009). However, it is important to verify whether the FXS allele results provided by the developed approach are reliable. An elegant way to reach this is by calculating the accuracy, sensitivity, and specificity of the proposed approach and comparing their results with those obtained by another method considered the gold standard. Interestingly, in regard to FXS, only few previous studies were found presenting that focus. Rosales-Reynoso *et al.* (2007) compared 29 results obtained by methylation-specific PCR with the SB analysis and found sensitivity and specificity values of 100%. High accuracy was found between the PCR method and SB, as reported by Filipovic-Sadic *et al.* (2010) and Chen *et al.* (2010). In the present study, comparing the Tassone *et al.* (2008) PCR-based screening method for FXS results with SB, when considering all types of FXS alleles (NL, PM, and FM), accuracy, sensitivity, and specificity demonstrated values of 100%. This result proves that the PCR-based screening method is able to detect the presence of all the FXS alleles, similarly as concluded by Tassone *et al.* (2008). Considering only the PM and NL alleles in the analysis, sensitivity and accuracy decreased to 88.6% and 89.5%, respectively (Fig. 4B). This occurred because that the PCR-based screening method failed to detect the real PM allele in four individuals that were incorrectly classified as FM (two men and two women, Table 1). The specificity remains at 100%, probably because the PCR was able to find all the three individuals carrying the NL alleles. It is known that when a test demonstrates high specificity, the sensitivity decreases because there is a potential presence of false negatives (Sabbatini, 1995); in this case, the

four PM alleles in the carriers were wrongly diagnosed by the PCR method as carriers of FM.

In the analysis performed on samples of subjects found as carrying the FM and NL alleles, besides sensitivity and accuracy, the specificity also decreased. The finding of four individuals who were found as FM allele carriers by the PCR method when they were actually carriers of PM alleles (by SB method, Fig. 4C-b) explains the specificity decrease. To show clear evidence, these individuals were the same represented in the Figure 4B-c. Similarly to this study, Filipovic-Sadic *et al.* (2010) also found that two individuals were found as FM carriers by the *FMRI* PCR method, while they were scored as carriers of PM alleles by using the SB method. The authors mentioned that an analytical titration of FM and NL genomic DNA templates demonstrated that PCR is fivefold more sensitive than SB for detecting the FM allele. Considering this, and similarly the results obtained for the two individuals investigated by Filipovic-Sadic *et al.* (2010), we wonder if the four individuals tested here as PM allele carriers by the SB method could actually carry both PM and a low abundance of FM alleles. Perhaps, using the methodology described by Filipovic-Sadic *et al.* (2010) or Chen *et al.* (2010), this problem can be solved.

Moreover, the PCR method was not able to detect allelic mosaicism as we can see in the Figure 4C-c. It is worth keeping in mind that this analysis considered only the FM and NL alleles; therefore, a false-negative result happens when someone is not found as the FM allele carrier when, in fact, this allele is present (even if he/she carries a PM allele). This occurred with the mosaic subject investigated here, which resulted in the false-negative outcome, since this subject was only found to be as the carrier of the PM allele by the PCR method, while by SB, he was also an FM allele carrier.

In conclusion, we reproduced the PCR-based screening method for FXS reported by Tassone *et al.* (2008), using only the Expand Long Template PCR System Enzyme. Analyses of accuracy, sensitivity, and specificity of the PCR-based screening method in comparison with SB showed that the PCR methodology employed here was able to identify 100% of the different FXS alleles. However, the differentiation between PM and FM alleles was not accurate. The final results indicate that the PCR method is useful as a screening method for FXS and can be executed by any molecular biology laboratory, since it deserves conventional equipment. Nevertheless, SB is still the gold-standard method for the molecular diagnosis of FXS, because it is able to identify the number of CGG repeats and the methylation status.

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### Author Disclosure Statement

No competing financial interests exist.

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