Songklanakarin J. Sci. Technol.
43 (3), 816-823, May - Jun. 2021

http://www.sjst.psu.ac.th

*Original Article*

# Development and validation of a single-tube multiplex PCR for rapid screening of Fragile X and Fragile XE syndromes of *FMR1* and *FMR2* genes

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Received: 31 March 2020; Revised: 7 June 2020; Accepted: 9 June 2020

**Abstract**

Fragile X (FRAXA) syndrome and fragile XE (FRAXE) syndrome are caused by the expansion of a trinucleotide repeat in the *FMR1* and *FMR2* genes, respectively. Currently, there are several methods available for fragile X syndrome screening in a large population, however these methods require relatively expensive equipment and have limitations in some laboratory settings. This study developed a multiplex PCR of triplet repeats in *FMR1* and *FMR2* genes using standard PCR instruments. The new multiplex PCR method was tested in known samples with variable repeat sizes of *FMR1* and *FMR2* genes to validate the technique and was then applied to prospective index male samples. All the multiplex PCR results matched well results from standard methods. We propose a single-tube multiplex PCR technique, which is very reliable for rapid screening *FMR1* and *FMR2* normal and expanded alleles in males for a large cohort study, and can be used in limited-resource settings.

**Keywords:** *FMR1*, *FMR2*, fragile X syndrome, multiplex PCR, screening

## 1. Introduction

Fragile X syndrome (FXS) is the most common cause of X-linked intellectual disability (ID). The two forms of fragile X syndrome, fragile X syndrome (FRAXA) and fragile XE syndrome (FRAXE), are caused by trinucleotide repeat expansion of CGG and CCG repeats at the 5' untranslated region of the *FMR1* and *FMR2* genes, respectively. Although these syndromes may be linked by the common mechanism of trinucleotide repeat expansion, they are different in prevalence and in molecular basis of the syndrome. The FRAXA syndrome is the most common form of familial ID with an incidence of 1 in 4,000 males and 1 in 8,000 females (Bailey *et al.*, 2017; Crawford, Acuña, & Sherman, 2001; Hill, Archibald, Cohen, & Metcalfe, 2010; Turner, Webb, Wake & Robinson, 1996), while the ID associated with the FRAXE syndrome is a rare syndrome with an incidence of ~1 in 50,000 Caucasian males, which is approximately 14-fold less than the incidence of FRAXA syndrome (Brown, 1996; Knight *et al.*, 1996). The molecular basis of FRAXA and FRAXE was elucidated by isolation of the CGG repeats of *FMR1* and CCG repeats of *FMR2* at the 5'untranslated regions (UTR) of these genes. The CGG repeats in *FMR1* can be classified into four categories: normal (5-44 CGG repeats), intermediate or grey zone (45-54 CGG repeats), premutation (55-200 CGG repeats) and full mutation

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(>200 CGG repeats) (Monaghan, Lyon & Spector, 2013). Individuals with *FMR2* are classified as having normal alleles (6-25 CCG repeats) or full mutation alleles (>200 CCG repeats). The FXS is caused by full mutation of either CGG or CCG repeats and subsequent methylation of the *FMR1* and *FMR2* genes (Gecz, 2000; Knight *et al.*, 1993). Furthermore, premutation (55-200 CGG repeats) of the *FMR1* gene is associated with an increased risk of developing fragile X-associated tremor/ataxia syndrome (FXTAS, OMIM 300623) and fragile X-associated primary ovarian insufficiency (FXPOI, OMIM 311360).

There are several techniques available for screening and diagnosis of FXS. Cytogenetic analysis was the original method for diagnosis of FXS, and was performed in low folate media and folate inhibitor such as trimethoprim, thymidine and FUdR as a fragile X inducer, which is then used to detect fragile sites on chromosome X (Sutherland, 1979; Sutherland, Baker, & Fratini, 1985). However, this technique is inadequate for carrier detection and cannot distinguish FRAXE fragile sites from FRAXA fragile sites, which are both located at Xq27.3-q28 (Knight *et al.*, 1996). Cytogenetic analysis is no longer used because it is less sensitive and more costly than molecular genetic testing and the method is replaced by combination of PCR and Southern blot analysis of *FMR1* mutations, which can differentiate between FRAXE and FRAXA. Conventional PCR can determine CGG repeat size ranging from the normal throughout small premutation allele, but larger expansions are refractory to PCR amplification due to their large amplicon sizes and high GC contents (Monaghan *et al.*, 2013). For Southern blot analysis, this technique can determine large expansion and methylation status; however, it is a costly and time-consuming procedure, requires a large amount of DNA, and provides inexact CGG sizing. Other methods for *FMR1* testing are also currently used, such as methylation-specific PCR (MS-PCR) (Weinhäusel & Haas, 2001; Zhou *et al.*, 2006), methylation-melting-curve PCR (Elias *et al.*, 2011; Teo, Rajan-Babu, Law, Lee, & Chong, 2013), and triplet-primed PCR (TP-PCR) (Chen *et al.*, 2010; Tassone, Pan, Amiri, Taylor, & Hagerman, 2008; Zhou *et al.*, 2006) and TP-PCR with melt curve analysis (TP-PCR MCA) (Tan, Lian, Faradz, Winarni, & Chong, 2018); however, these methods have limitations, such as requirement of an expensive automatic system and relatively moderate to high costs.

To attempt to overcome the limited-resource-setting mismatch with the available methods for detecting FRAXA and FRAXE, we developed an alternative multiplex PCR method using triplet repeats in the *FMR1* and *FMR2* genes. The benefits of using multiplex PCR to screen FXS in a large population are that this method is easy to perform, and no expensive equipment is required, which makes it suitable for low income countries.

## 2. Materials and Methods

### 2.1 Subjects

A total of 101 Thai male patients with intellectual disability of unknown cause were recruited from the Rajanagarindra Institute of Child Development in Chiangmai, Thailand, with ages ranging from 1 to 15 years at the time of recruitment. Out of the 101 male patients who enrolled in this study, 98 patients were previously evaluated using a five-item clinical checklist for FXS, including family history, long and narrow face, prominent and large ears, attention deficit/hyperactivity, and testicular volume (Limprasert *et al.*, 2000). For the development of single-tube multiplex PCR, we selected males with variable *FMR1* CGG repeat sizes depending on the available DNA samples including normal (29, 30, 36, 43, 44 CGG), intermediate (47, 52 CGG), premutation (90 CGG), and full mutation, to validate the technique. Although, we aim to use the developed technique to screen male cases, we also selected females with normal and premutation of the *FMR1* and *FMR2* to test our technique in this step.

The study was approved by the Institutional Ethics Committee of the Faculty of Medicine, Prince of Songkla University Thailand (SUB.EC 47/364-007 and 49/364-010) and informed consent was obtained from the parent or parents of the enrolled children.

### 2.2 Development of single-tube multiplex PCR of *FMR1*, *FMR2* and *SRY* genes

DNA was extracted from whole blood samples using the standard phenol-chloroform method (Green & Sambrook, 2012) with some slight modifications of centrification steps. Multiplex PCR was performed to amplify the *FMR1*, *FMR2* and *SRY* genes in a final reaction volume of 20 µl. *SRY* gene was used as an internal control for sex identification. The PCR reaction includes 1X IMMOLASE$^{TM}$ buffer (Bioline), 100 ng genomic DNA, 1.5 mM $MgCl_2$, 200 µM each of dATP, dCTP, and dTTP, 100 µM of dGTP, 100 µM 7-deaza dGTP, 2.2 M betaine, 0.25 µM of *SRY* forward primer and *SRY* reverse primer (Cui, Warnes, Jeffrey, & Matthews, 1994), 0.25 µM of FRAXE forward primer and FRAXE reverse primer (Knight *et al.*, 1993), 0.5 µM of FRAXA forward primer and FRAXA reverse primer (Chong, Eichler, Nelson, & Hughes, 1994), and 1U of IMMOLASE$^{TM}$ DNA polymerase (Bioline). The primer sequences used in the study are shown in Table 1. The PCR reactions were carried out beginning with an initial hot start at 95°C for 10 minutes,

Table 1. Primers for the multiplex PCR used in the study

| Gene | Primer | Primer sequence | Amplified fragment length (bp) | References |
|---|---|---|---|---|
| *SRY* | Forward | 5'-CAT GAA CGC ATT CAT CGT GTG GTC-3' | 254 | Cui *et al.*, |
| (Internal control) | Reverse | 5'-CTG CGG GAA GCA AAC TGC AAT TCT T-3' | | 1994 |
| FRAXE | Forward | 5'-AAG CGG CAG TGG CAC TGG GC-3' | 385 | Knight *et al.*, |
| *(FMR2)* | Reverse | 5'-CGC CCC CTG TGA GTG TGT AAG TGT GTG ATG-3' | | 1993 |
| FRAXA | Forward | 5'-CAG CGT TGA TCA CGT GAC GTG GTT TCA GTG-3' | 430 | Chong *et al.*, |
| *(FMR1)* | Reverse | 5'-GAT GGG GCC TGC CCT AGA GCC AAG TAC-3' | | 1994 |

followed by 30 cycles of denaturation at 95°C for 1 minute, annealing at 64°C for 20 seconds, and extension at 72°C for 20 seconds, with a final extension of 72°C for 10 minutes. Subsequently, the PCR products were separated on 2.5% agarose gel and visualized through ethidium bromide staining under a UV transilluminator.

To validate the results from the developed multiplex PCR, we used conventional fluorescent PCR, MS-PCR (Charalsawadi, Sripo, & Limprasert, 2005) and/or Southern blot analysis described elsewhere. Briefly, for Southern blot analysis, 7-10 μg of genomic DNA was digested with EcoRI and EagI restriction enzymes. Digested genomic DNA was separated on 0.8% agarose gel containing Tris-acetate-EDTA buffer and then transferred to membrane. The membrane was then hybridized with a *FMR1*-specific genomic probe (StB12.3), which was used to detect 5′ UTR of *FMR1* gene (Limprasert, Ruangdaraganon, Sura, Vasiknanonte, & Jinorose, 1999).

### 2.3 Fluorescent multiplex PCR

The primers of developed multiplex PCR in this study can also be used for fluorescent PCR. Primers used for fluorescent multiplex PCR were labeled with different dye colors dependent on PCR product size. Fluorescent multiplex PCR was performed to amplify the *FMR1*, *FMR2* and *SRY* genes in a final reaction volume of 10 μl. *SRY* gene was used as an internal control. The PCR reaction mixes contain 1X IMMOLASE$^{TM}$ buffer (Bioline), 25 ng genomic DNA, 1.5 mM $MgCl_2$, 200 μM each of dATP, dCTP, dTTP and 7-deaza dGTP, 2.2 M betaine, 0.4 μM of *SRY* forward 5'FAM-labeled primer and *SRY* reverse primer, 0.4 μM of FRAXE forward 5'FAM-labeled primer and FRAXE reverse primer, 0.2 μM of FRAXA forward 5'VIC-labeled primer and FRAXA reverse primer, and 1U of IMMOLASE$^{TM}$ DNA polymerase (Bioline). The PCR reactions were carried out beginning with an initial hot start at 95°C for 10 minutes, followed by 35 cycles of denaturation at 95°C for 1 minute, annealing at 64°C for 1 minute, and extension at 72°C for 1 minute, with a final extension of 72°C for 10 minutes. One microliter of the PCR products was added to 10.7 μl Hi-Di formamide and 0.3 μl GeneScan 500 Liz Size standard after heating at 95°C for 2 minutes, and the mixtures were applied using ABI 3500 Genetic Analyzer (Applied Biosystems). Electropherograms were analyzed using GeneMapper v5.0 software (Applied Biosystems).

## 3. Results

### 3.1 Development of single-tube multiplex PCR of *FMR1*, *FMR2* and *SRY* genes

For the best amplification of the *FMR1*, *FMR2* and *SRY* genes in the multiplex PCR reactions, all important factors including the various PCR enhancers (DMSO, BSA or betaine), annealing temperature, ratios of the *FMR*1, *FMR2* and *SRY* primer concentrations and several commercial Taq DNA polymerases were optimized. We found that the IMMOLASE$^{TM}$ DNA polymerase was the best enzyme for PCR amplification with non-specific amplicons, and betaine at 2.2 M was the optimum concentration enhancer for performing multiplex PCR at a 64°C annealing temperature with a primer ratio of *FMR*1, *FMR2* and *SRY* of 2:1:1. The expected PCR products for normal sample CGG or CCG repeats in the *FMR1* and *FMR2* genes were 355-502 bp (5-54 CGG repeats) and 303-385 bp (3-30 CCG repeats), respectively. The PCR product of the *SRY* gene used as an internal control was 254 bp. The normal female and *FMR2* carrier female showed both *FMR1* and *FMR2* heterozygous alleles, but absence of the *SRY* gene. The normal males showed both *FMR1* and *FMR2* hemizygous alleles and always showed the PCR product of *SRY* gene as an internal control. Of note, when a sample revealed the absence of *FMR1* or *FMR2* alleles of multiplex PCR indicating positive FXS results and presence of *SRY* allele in males as an internal control.

To prove the efficiency of the developed method, the optimized multiplex PCR conditions were tested in known samples with variable repeat sizes of the *FMR1* and *FMR2* gene. The maximum number of CGG repeats of *FMR1* that can be amplified by multiplex PCR in available known DNA samples was 52 repeats. Samples with high-range premutation or full mutation allele of *FMR1* and *FMR2* were not amplified by this method (Figure 1), thus the subsequent workup by standard methods including MS-PCR and/or Southern blot analysis was further processed for confirmation of FXS. The results of developed multiplex PCR of all tested samples were consistent with conventional fluorescent PCR, MS-PCR and/or Southern blot analysis.

For fluorescent multiplex PCR, the primer sets of multiplex PCR were labelled with fluorescent dyes and applied for fluorescent PCR as shown in Figure 2. The labelled PCR products for normal sample CGG or CCG repeats in the *FMR1* and *FMR2* genes were approximately 346-493 bp (5-54 CGG repeats) and 289-370 bp (3-30 CCG repeats), respectively. The labelled PCR product of the *SRY* gene used as an internal control in case of negative amplification of the *FMR1* and *FMR2* genes was approximately 248 bp. Note that sizes of the fluorescent PCR products did not match exactly because a size standard was used to extrapolate the base-pair sizes of the sample product peaks. The mobility of labeled fragments was affected by the sequence composition, the fluorescent label, and electrophoresis conditions.

### 3.2 Validation of the developed multiplex PCR

After PCR was performed under optimal conditions in selected known samples to validate the accuracy of this technique, we further tested this method under the same conditions by screening prospective samples from 101 males with ID of unknown FXS status. The results showed 93 samples with normal repeats of *FMR1* (93/101 samples) and 101 samples with normal repeats of *FMR2* (101/101 samples). Samples with no *FMR1* allele amplification by our developed method were further analyzed by MS-PCR and Southern blot analysis, which detected eight males (8/101, 7.9%) with full mutation of the *FMR1* gene. All the multiplex PCR results matched well the results from conventional PCR, MS-PCR and/or Southern blot analysis. Thus, the sensitivity and specificity of the developed multiplex PCR for screening *FMR1* normal and expanded alleles in available samples were 100%. However, no expanded *FMR2* alleles were detected in this study. The workflow of the study is shown in Figure 3.

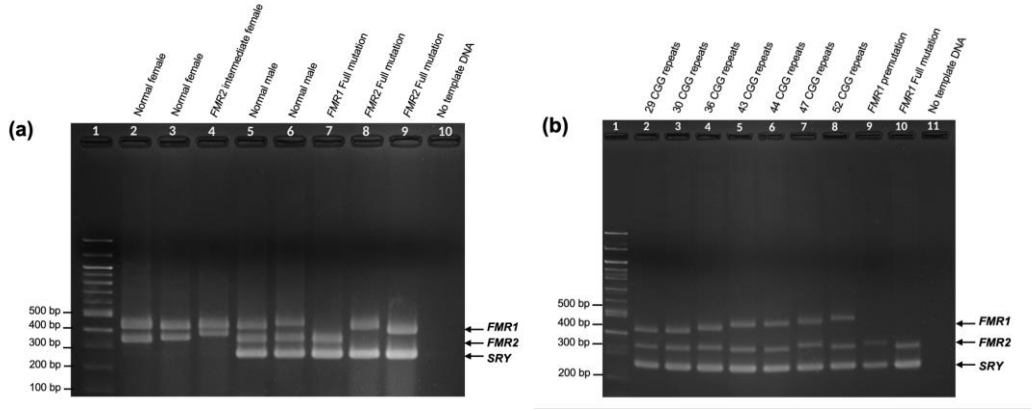


Figure 1. Results of single-tube multiplex PCR for the *FMR1*, *FMR2* and *SRY* genes. (a) Normal female (lane 2: 29,29 CGG *FMR1* alleles and 17,18 CCG *FMR2* alleles; lane 3: 27,28 CGG *FMR1* alleles and 21,22 CCG *FMR2* alleles) and *FMR2* intermediate female (lane 4: 31,31 CGG *FMR1* alleles and 30,31 CCG *FMR2* alleles). Normal males (lane 5: 29 CGG *FMR1* allele and 18 CCG *FMR2* allele; lane 6: 36 CGG *FMR1* allele and 20 CCG *FMR2* alleles). Males with *FMR1* full mutation (lane 7) and *FMR2* full mutation (lane 8 and 9) (b) Multiplex PCR in males with focusing on variable repeats of *FMR1*. Lane 1: 100 bp marker; Lane 2-6: normal male (lane 2: 29 CGG *FMR1* allele and 18 CCG *FMR2* allele; lane 3: 30 CGG *FMR1* allele and 18 CCG *FMR2* allele; lane 4: 36 CGG *FMR1* allele and 20 CCG *FMR2* allele; lane 5: 43 CGG *FMR1* allele and 18 CCG *FMR2* allele; lane 6: 44 CGG *FMR1* allele and 18 CCG *FMR2* allele); Lane 7-8: *FMR1* intermediate males (lane 7: 47 CGG *FMR1* allele and 23 CCG *FMR2* allele; lane 8: 52 CGG *FMR1* allele and 19 CCG *FMR2* allele); Lane 9: *FMR1* premutation male (90 CGG *FMR1* allele and 24 CCG *FMR2* allele); Lane 10: *FMR1* full mutation male (>200 *FMR1* CGG repeats and 18 CCG *FMR2* allele); Lane 11: no template DNA. Note that the positive and carrier DNA samples of *FMR2* used in this study were provided by Professor Dr. Jozef Gecz.

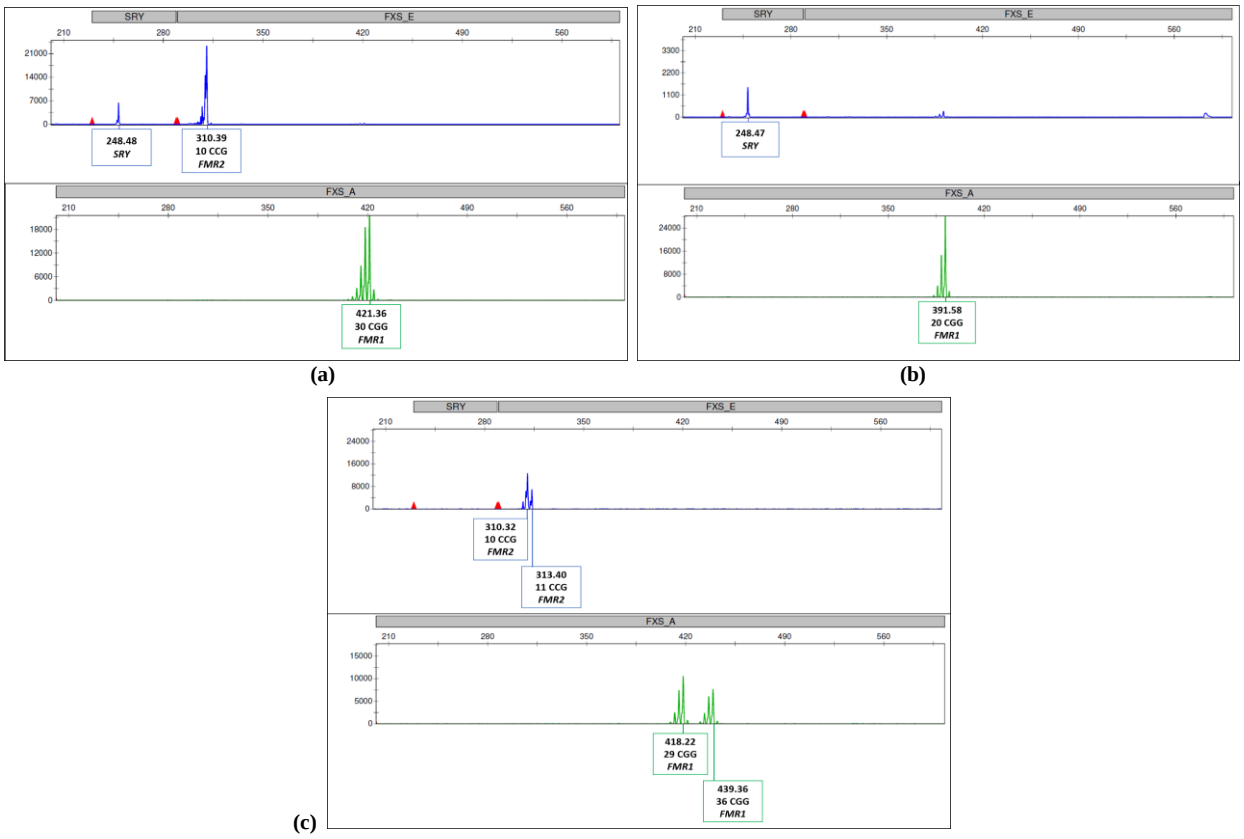


Figure 2. Fragment analysis result of fluorescent multiplex PCR for the *FMR1*, *FMR2* and *SRY* genes from male and female samples. (a) A normal male shows the peaks of 248 bp *SRY*, 10 CCG repeats of *FMR2* (upper panel) and 30 CGG *FMR1* alleles (lower panel). (b) A male with *FMR2* mutation shows the peaks of 248 bp *SRY* allele without *FMR2* (upper panel) and 20 CGG *FMR1* allele (lower panel). (c) A normal female shows the peaks of 10, 11 CCG *FMR2* alleles without *SRY* allele (upper panel) and 29, 36 CGG *FMR1* alleles (lower panel). Note that sizes of the fluorescent PCR products do not match exactly because different dye labels are used with PCR product and internal size standard thus impacting their relative mobilities.

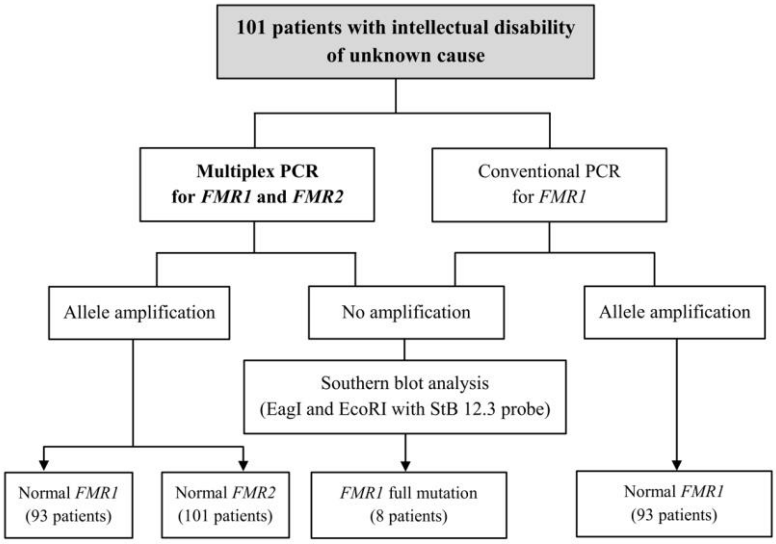


Figure 3. Schematic diagram of the study design

## 4. Discussion

In the present study, we developed a single-tube multiplex PCR, which is a rapid and cost-effective alternative for fragile X and fragile XE syndromes screening in males. This developed method has given an impetus to distinguish male with normal allele from expanded allele (premutation or full mutation) in large-scale or population-based high risk screening. The gold-standard methods for the FXS diagnosis use a combination of conventional PCR and Southern blot analysis, but this combined method is not suitable for large-scale screening because it is a costly and time-consuming procedure that requires large amount of template DNA. Nowadays, there are several alternative PCR-based approaches that have been developed for fragile X syndrome (FRAXA) screening (Hantash *et al.*, 2010; Lyon *et al.*, 2010; Rajan-Babu, Law, Yoon, Lee, & Chong, 2015; Strom *et al.*, 2007; Tan *et al.*, 2018; Tassone *et al.*, 2008; Teo, Law, Lee, & Chong, 2012). Most of these methods require the use of expensive equipment and high technology instrumentation, especially a capillary electrophoresis instrument, and have only focused on detecting *FMR1* CGG repeat expansion. However, a recent study demonstrated that the low-cost PCR was able to amplify *FMR1* alleles with large CGG repeats, but the procedure was complex and time-consuming, especially in the restriction enzyme digestion step (Hayward, Zhou, Kumari, & Usdin, 2016). For fragile XE syndrome, molecular diagnostic techniques still use conventional PCR and fluorescent PCR, and new methods for fragile XE syndrome diagnosis have not been developed for a long time (Barros Santos & Gonçalves Pimentel, 2003; Hećimović, Tarnik, Barić, Cakarun, & Pavelić, 2002; Katikala *et al.*, 2011; Murray *et al.*, 1996; Santos, Costa Lima, & Pimentel, 2001). To overcome these limitations, we have developed the single-tube multiplex PCR for rapid screening of FXS in both the *FMR1* and *FMR2* genes. This method is rapid and able to flag the presence of normal and expanded alleles, and it only requires basic equipment including a conventional thermal cycler and a gel documentation system, which are available in most laboratories.

The single-tube multiplex PCR method identified all male samples with normal repeats of *FMR1* and *FMR2*, corresponding to conventional fluorescent PCR. Because the multiplex PCR method was unable to distinguish between male with premutation and male with full mutation allele that were not amplified by PCR: samples with no allele amplification by this method indicate expanded alleles (premutation or full mutation) were further analyzed by MS-PCR and Southern blot analysis. The frequency of FXS in Thai males with ID of unknown cause in this study was 7.9%, which is similar to the frequency of FXS in Thai males with developmental delay (DD) of unknown cause reported in a previous study (Limprasert *et al.*, 1999), and is also similar to previous studies in patients with DD and ID in other populations including Southeast and South Asian populations (Ali *et al.*, 2017; Chowdhury *et al.*, 2006; Fatima *et al.*, 2014; Sharma, Gupta, & Thelma, 2001; Winarni, Utari, Mundhofir, Mundhofir, & Faradz, 2013). This study found no expansion of a CCG repeat in the *FMR2* detected by multiplex PCR, which is similar to the frequencies of FRAXE syndrome reported in previous studies conducted in several populations. The full mutation of the *FMR2* was estimated to have a rate of 0.14% (3/2,184) in male patients with DD and suspicion of FXS (Brown, 1996), and another study reported the frequency of full mutations in *FMR2* patients to be 0.55% (1/182) in males from Spanish special schools (Milà *et al.*, 1997). However, the full mutation of *FMR2* has not been reported in Thailand. The frequency of *FMR2* has been reported to be much less than *FMR1* (~14-fold) (Knight *et al.*, 1996), so it is not surprising that no expansion of CCG repeats in *FMR2* was found in this study. For the distribution of CCG repeats in *FMR2*, the most frequent alleles in the Thai population were in the normal range including 14, 15 and 18 CCG repeats (Richards *et al.*, 1996), however these data may not represent the actual number of CCG repeats because of technical limitations at that time. Moreover, the high number of CCG repeats in *FMR2* overlap with the low number of CGG repeats in *FMR1*, they are very rare because they are both very uncommon alleles (< 1%). Due to the limited sample size in this study, further studies with larger sample count using this

new multiplex PCR are required to verify the prevalence of *FMR1* and *FMR2* full mutations in Thailand. Because the CCG repeat expansion in *FMR2* is considered rare, the primary benefit of the developed PCR in this study is in the screening for *FMR1* mutation. However, we recommend for use the single-tube multiplex PCR method developed in this study in initial population screening, because it was able to amplify both *FMR1* and *FMR2* genes cost-effectively.

Based on our observation, the multiplex PCR cannot distinguish a homozygote normal female from a full mutation female because both cases show only one PCR product band of *FMR1* and *FMR2* genes each. This problem is due to preferential amplification of the smaller sized allele, allowing false negative result for an affected female, thus this approach is not suitable for female FXS screening. However, the majority of FXS cases are males, thus this limitation is not a major problem in FXS screening. Of note, because of limitations of available male samples with variable repeats, we cannot evaluate the performance in individuals with low premutation range from 55 to 89 CGG repeats of *FMR1* or with premutation of *FMR2*. Another limitation is that the mosaic normal/premutation and the mosaic normal/full mutation are misdiagnosed as normal, because only the normal allele could be detected in this PCR. However, mosaic patients are apparently rare and might not be a significant cause of error in large population screenings.

The developed multiplex PCR is used as a qualitative assessment method for rapid screening of repeat status, and it is not designed for repeat sizing, especially of premutation or full-mutation alleles; thus, all samples with no allele amplification by this method should be subjected to methylation status and CGG repeat sizing for verification of expansion status using standard methods. Comparison of our multiplex PCR with other methods commonly used for testing FXS is shown in Table 2. The multiplex PCR method developed in this study is suitable for large-scale screening of expanded alleles of both *FMR1* and *FMR2* genes in males, especially in developing countries that lack advanced molecular facilities, because the method can be performed with standard laboratory equipment. Although other assays exist that can be used for *FMR1* screening in a cost-effective manner, the usefulness of the developed method lies in its ability to simultaneously screen for expansions in both *FMR1* and *FMR2* genes. Moreover, this method may be used for FXS screening with a small amount of DNA from blood spots cards when more validation is required, and the primer sets used in this study can also be applied for fluorescent PCR.

## 5. Conclusions

The proposed method of using multiplex PCR of triplet repeats in the *FMR1* and *FMR2* genes can be useful as an alternative assay for rapid screening of male patients suspected of having FXS in both the *FMR1* and *FMR2* genes. The ability to screen for both *FMR1* and *FMR2* mutations simultaneously in patients with ID and DD is a useful saving both time and resources. This method is easy to interpret and implement in clinical investigations by laboratories with limited equipment. The developed technique has the advantages of allowing to screen a large number of samples at a low cost and with less time consumption than the standard methods, and it is suitable for low-income countries. As an additional benefit, in places where there is no problem with diagnostic facilities, the primers labelled with fluorescent tags for multiplex PCR could be convenient for use with fluorescent PCR.

## Acknowledgements

The authors are thankful to Professor Dr. William Ted Brown for kindly providing the StB12.3 probe and the positive DNA samples of the *FMR2*. We are also thankful to Professor Dr. Jozef Gecz for providing the positive and carrier DNA samples of *FMR2.* We thank Dr. Thanya Sripo for technical supervision of the multiplex PCR development.

This study was supported by grants from the Faculty of Medicine, Prince of Songkla University (nos. 47/364-007 and 49/364-010).

## References

Ali, E. Z., Yakob, Y., Md Desa, N., Ishak, T., Zakaria, Z., Ngu, L. K., & Keng, W. T. (2017). Molecular analysis of fragile X syndrome (FXS) among Malaysian patients with developmental disability. *The Malaysian Journal of Pathology*, *39*(2), 99–106.

Table 2. Comparison of a single-tube multiplex PCR with the most common methods used for FXS testing.

| | Conventional fluorescent PCR | Methylation PCR | Triplet primed PCR | Southern blot (Gold standard) | Multiplex PCR (This study) |
|---|---|---|---|---|---|
| Time required | ~ 4 hours | ~ 24-48 hours | ~ 4-6 hours | 4-5 days | ~ 4 hours |
| Labor | Minimal | Moderate | Minimal | Intensive | Minimal |
| DNA amount | 0.025 µg | 0.025-0.1 µg | 0.1 µg | 5-10 µg | 0.1 µg |
| Essential equipment/ reagent | PCR machine Genetic Analyzer | PCR machine | PCR machine Genetic Analyzer | Chemiluminescent Kit, X-ray film | PCR machine |
| Relative cost | Moderate | Low | Moderate | High | Low |
| Detection of CGG repeats of *FMR1* | Yes (Normal to premutation) | No | Yes (Normal to full mutation) | Yes (Approximate size) | Distinguish between normal and expanded alleles of *FMR1* and *FMR2* |
| Methylation status | No | Yes | No | Yes | No |

Bailey, D. B., Berry-Kravis, E., Gane, L. W., Guarda, S., Hagerman, R., Powell, C. M., . . . Wheeler, A. (2017). Fragile X newborn screening: Lessons learned from a multisite screening study. *Pediatrics*, *139*(Supplement 3), S216–S225. doi: 0.1542/peds. 2016-1159H

Barros Santos, C., & Gonçalves Pimentel, M. M. (2003). The influence of expanded unmethylated alleles for FRAXA/FRAXE loci in the intellectual performance among Brazilian mentally impaired males. *International Journal of Molecular Medicine*, *12*(3), 385–389.

Brown, W. T. (1996). The FRAXE syndrome: Is it time for routine screening? *American Journal of Human Genetics*, *58*(5), 903–905.

Charalsawadi, C., Sripo, T., & Limprasert, P. (2005). Multiplex methylation specific PCR analysis of fragile X syndrome: Experience in Songklanagarind Hospital. *Journal of the Medical Association of Thailand*, *88*(8), 1057–1061.

Chen, L., Hadd, A., Sah, S., Filipovic-Sadic, S., Krosting, J., Sekinger, E., . . . Latham, G. J. (2010). An information-rich CGG repeat primed PCR that detects the full range of fragile X expanded alleles and minimizes the need for southern blot analysis. *The Journal of Molecular Diagnostics*, *12*(5), 589–600. doi:10.2353/jmoldx.2010.090227

Chong, S. S., Eichler, E. E., Nelson, D. L., & Hughes, M. R. (1994). Robust amplification and ethidium-visible detection of the fragile X syndrome CGG repeat using Pfu polymerase. *American Journal of Medical Genetics*, *51*(4), 522–526. doi:10.1002/ajmg.13205 10447

Chowdhury, M. R., Kabra, M., Sharma, D., Singh, D., Dabral, A., Thelma, B. K., & Kalra, V. (2006). Fragile X screening for FRAXA and FRAXE mutations using PCR based studies: Results of a five year study. *Indian Journal of Human Genetics*, *12*(1). Retrieved from https://tspace.library.utoronto.ca/handle/1807/5958

Crawford, D. C., Acuña, J. M., & Sherman, S. L. (2001). FMR1 and the fragile X syndrome: Human genome epidemiology review. *Genetics in Medicine*, *3*(5), 359–371.

Cui, K. H., Warnes, G. M., Jeffrey, R., & Matthews, C. D. (1994). Sex determination of preimplantation embryos by human testis-determining-gene amplification. *Lancet (London, England)*, *343* (8889), 79–82. doi:10.1016/s0140-6736(94)90815-x

Elias, M. H., Ankathil, R., Salmi, A. R., Sudhikaran, W., Limprasert, P., & Zilfalil, B. A. (2011). A new method for FMR1 gene methylation screening by multiplex methylation-specific real-time polymerase chain reaction. *Genetic Testing and Molecular Biomarkers*, *15*(6), 387–393. doi:10.1089/gtmb. 2010.0191

Fatima, T., Zaidi, S. A. H., Sarfraz, N., Perween, S., Khurshid, F., & Imtiaz, F. (2014). Frequency of FMR1 gene mutation and CGG repeat polymorphism in intellectually disabled children in Pakistan. *American Journal of Medical Genetics, Part A*, *164A*(5), 1151–1161. doi:10.1002/ajmg.a.36423

Gecz, J. (2000). The FMR2 gene, FRAXE and non-specific X-linked mental retardation: Clinical and molecular aspects. *Annals of Human Genetics*, *64*(Pt 2), 95–106. doi:10.1017/S0003480000007983

Green, M. R., & Sambrook, J. (2012). *Molecular cloning: A laboratory manual* (4th ed.). New York, NY: Cold Spring Harbor Laboratory Press.

Hantash, F. M., Goos, D. G., Tsao, D., Quan, F., Buller-Burckle, A., Peng, M., . . . Strom, C. M. (2010). Qualitative assessment of FMR1 (CGG)n triplet repeat status in normal, intermediate, premutation, full mutation, and mosaic carriers in both sexes: Implications for fragile X syndrome carrier and newborn screening. *Genetics in Medicine*, *12*(3), 162–173. doi:10.1097/GIM.0b013e3181d0d40e

Hayward, B. E., Zhou, Y., Kumari, D., & Usdin, K. (2016). A set of assays for the comprehensive analysis of FMR1 alleles in the fragile X-related disorders. *The Journal of Molecular Diagnostics*, *18*(5), 762–774. doi:10.1016/j.jmoldx.2016.06.001

Hećimović, S., Tarnik, I. P., Barić, I., Cakarun, Z., & Pavelić, K. (2002). Screening for fragile X syndrome: Results from a school for mentally retarded children. *Acta Paediatrica*, *91*(5), 535–539. doi:10. 1080/080352502753711650

Hill, M. K., Archibald, A. D., Cohen, J., & Metcalfe, S. A. (2010). A systematic review of population screening for fragile X syndrome. *Genetics in Medicine*, *12*(7), 396–410. doi:10.1097/GIM.0b013e3181e38fb6

Katikala, L., Guruju, M. R., Madireddi, S., Vallamkonda, O., Vallamkonda, N., Persha, A., & Spurgeon, A. M. P. J. (2011). Distribution of CGG/GCC repeats at the FMR1 and FMR2 genes in an Indian population with mental retardation of unknown etiology. *Genetic Testing and Molecular Biomarkers*, *15*(4), 281–284. doi:10.1089/gtmb.2010.0167

Knight, S. J., Flannery, A. V., Hirst, M. C., Campbell, L., Christodoulou, Z., Phelps, S. R., . . . Pembrey, M. E. (1993). Trinucleotide repeat amplification and hypermethylation of a CpG island in FRAXE mental retardation. *Cell*, *74*(1), 127–134. doi:10.10 16/0092-8674(93)90300-f

Knight, S. J., Ritchie, R. J., Chakrabarti, L., Cross, G., Taylor, G. R., Mueller, R. F., . . . Davies, K. E. (1996). A study of FRAXE in mentally retarded individuals referred for fragile X syndrome (FRAXA) testing in the United Kingdom. *American Journal of Human Genetics*, *58*(5), 906–913.

Limprasert, P., Ruangdaraganon, N., Sura, T., Vasiknanonte, P., & Jinorose, U. (1999). Molecular screening for fragile X syndrome in Thailand. *The Southeast Asian Journal of Tropical Medicine and Public Health*, *30*(Supplement 2), 114–118.

Limprasert, P., Ruangdaraganon, N., Vasiknanonte, P., Sura, T., Jaruratanasirikul, S., Sriwongpanich, N., & Sriplung, H. (2000). A clinical checklist for fragile X syndrome: Screening of Thai boys with developmental delay of unknown cause. *Journal of the Medical Association of Thailand*, *83*(10), 1260–1266.

Lyon, E., Laver, T., Yu, P., Jama, M., Young, K., Zoccoli, M., & Marlowe, N. (2010). A simple, high-throughput assay for Fragile X expanded alleles using triple repeat primed PCR and capillary electrophoresis. *The Journal of Molecular Diagnostics*, *12*(4), 505–511. doi:10.2353/jmoldx.2010.090229

Milà, M., Sànchez, A., Badenas, C., Brun, C., Jiménez, D., Villa, M. P., . . . Estivill, X. (1997). Screening for FMR1 and FMR2 mutations in 222 individuals from Spanish special schools: Identification of a case of FRAXE-associated mental retardation. *Human Genetics*, *100*(5–6), 503–507. doi:10.1007/s00439 0050542

Monaghan, K. G., Lyon, E., Spector, E. B., & American College of Medical Genetics and Genomics. (2013). ACMG standards and guidelines for fragile X testing: A revision to the disease-specific supplements to the standards and guidelines for clinical genetics laboratories of the American College of Medical Genetics and Genomics. *Genetics in Medicine*, *15*(7), 575–586. doi:10.1038/ gim.2013.61

Murray, A., Youings, S., Dennis, N., Latsky, L., Linehan, P., McKechnie, N., . . . Jacobs, P. (1996). Population screening at the FRAXA and FRAXE loci: Molecular analyses of boys with learning difficulties and their mothers. *Human Molecular Genetics*, *5*(6), 727–735. doi:10.1093/hmg/5.6.727

Rajan-Babu, I.-S., Law, H.-Y., Yoon, C.-S., Lee, C. G., & Chong, S. S. (2015). Simplified strategy for rapid first-line screening of fragile X syndrome: Closed-tube triplet-primed PCR and amplicon melt peak analysis. *Expert Reviews in Molecular Medicine*, *17*, e7. doi:10.1017/erm.2015.5

Richards, R. I., Crawford, J., Narahara, K., Mangelsdorf, M., Friend, K., Staples, A., . . . Sutherland, G. R. (1996). Dynamic mutation loci: Allele distributions in different populations. *Annals of Human Genetics*, *60*(5), 391–400. doi:10.1111/j.1469-1809.1996.tb 00437.x

Santos, C. B., Costa Lima, M. A., & Pimentel, M. M. (2001). A new PCR assay useful for screening of FRAXE/FMR2 mental impairment among males. *Human Mutation*, *18*(2), 157–162. doi:10.1002/ humu.1165

Sharma, D., Gupta, M., & Thelma, B. K. (2001). Expansion mutation frequency and CGG/GCC repeat polymorphism in FMR1 and FMR2 genes in an Indian population. *Genetic Epidemiology*, *20*(1), 129–144. doi:10.1002/1098-2272(200101)20:1<129 ::AID-GEPI11>3.0.CO;2-2

Strom, C. M., Huang, D., Li, Y., Hantash, F. M., Rooke, J., Potts, S. J., & Sun, W. (2007). Development of a novel, accurate, automated, rapid, high-throughput technique suitable for population-based carrier screening for Fragile X syndrome. *Genetics in Medicine*, *9*(4), 199–207. doi:10.1097/gim.0b013 e31803d3ac9

Sutherland, G. R. (1979). Heritable fragile sites on human chromosomes I. Factors affecting expression in lymphocyte culture. *American Journal of Human Genetics*, *31*(2), 125–135.

Sutherland, G. R., Baker, E., & Fratini, A. (1985). Excess thymidine induces folate sensitive fragile sites. *American Journal of Medical Genetics*, *22*(2), 433–443. doi:10.1002/ajmg.1320220234

Tan, V. J., Lian, M., Faradz, S. M. H., Winarni, T. I., & Chong, S. S. (2018). A single common assay for robust and rapid fragile X mental retardation syndrome screening from dried blood spots. *Frontiers in Genetics*, *9*, 582. doi:10.3389/fgene. 2018.00582

Tassone, F., Iong, K. P., Tong, T.-H., Lo, J., Gane, L. W., Berry-Kravis, E., . . . Hagerman, R. J. (2012). FMR1 CGG allele size and prevalence ascertained through newborn screening in the United States. *Genome Medicine*, *4*(12), 100. doi:10.1186/gm401

Tassone, F., Pan, R., Amiri, K., Taylor, A. K., & Hagerman, P. J. (2008). A rapid polymerase chain reaction-based screening method for identification of all expanded alleles of the fragile X (FMR1) gene in newborn and high-risk populations. *The Journal of Molecular Diagnostics*, *10*(1), 43–49. doi:10.2353/ jmoldx.2008.070073

Teo, C. R. L., Law, H.-Y., Lee, C. G., & Chong, S. S. (2012). Screening for CGG repeat expansion in the FMR1 gene by melting curve analysis of combined 5' and 3' direct triplet-primed PCRs. *Clinical Chemistry*, *58*(3), 568–579. doi:10.1373/clinchem.2011.174615

Teo, C. R. L., Rajan-Babu, I.-S., Law, H.-Y., Lee, C. G., & Chong, S. S. (2013). Methylation-specific triplet-primed PCR and melting curve analysis as a rapid screening tool for identifying actionable FMR1 genotypes. *Clinical Chemistry*, *59*(11), 1668–1670. doi:10.1373/clinchem.2013.206771

Turner, G., Webb, T., Wake, S., & Robinson, H. (1996). Prevalence of fragile X syndrome. *American Journal of Medical Genetics*, *64*(1), 196–197. Retrieved from https://doi.org/10.1002/(SICI)1096-8628(19960712)64:1<196::AID-AJMG35>3.0.CO;2-G

Weinhäusel, A., & Haas, O. A. (2001). Evaluation of the fragile X (FRAXA) syndrome with methylation-sensitive PCR. *Human Genetics*, *108*(6), 450–458. doi:10.1007/s004390100519

Winarni, T. I., Utari, A., Mundhofir, F. E. P., Hagerman, R. J., & Faradz, S. M. H. (2013). Fragile X syndrome: Clinical, cytogenetic and molecular screening among autism spectrum disorder children in Indonesia. *Clinical Genetics*, *84*(6), 577–580. doi:10.1111/cge.12095

Zhou, Y., Lum, J. M. S., Yeo, G.-H., Kiing, J., Tay, S. K. H., & Chong, S. S. (2006). Simplified molecular diagnosis of fragile X syndrome by fluorescent methylation-specific PCR and GeneScan analysis. *Clinical Chemistry*, *52*(8), 1492–1500. doi:10.1373/ clinchem.2006.068593