Validation of Methylation-specific Triplet-primed PCR Method for Fragile X Syndrome Diagnosis at Songklanagarind Hospital

การตรวจสอบความใช้ได้ของวิธีเมทิลเลชันสเปคซิฟิคทริพเพลทไพร์มพีซีอาร์สำหรับตรวจวินิจฉัยกลุ่มอาการโครโมโซมเอกซ์เปราะในโรงพยาบาลสงขลานครินทร์

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ABSTRACT

This study aimed to validate performance of methylation-specific triplet-primed PCR (msTP-PCR) method for detecting CGG expansion and methylation status of fragile X mental retardation-1 (*FMR1*) gene using male and female samples received from Songklanagarind Hospital. The procedures started by bisulfite modification of genomic DNA, followed by msTP-PCR amplification and capillary electrophoresis analysis. The results showed that all tested samples were correctly detected and corresponded to previous results from conventional PCR and/or Southern blot analysis. Therefore, this msTP-PCR method could be an alternative method for FXS diagnosis in the routine laboratory at Songklanagarind Hospital, which can decrease the requirement of Southern blot analysis.

บทคัดย่อ

การศึกษานี้มีวัตถุประสงค์เพื่อตรวจสอบประสิทธิภาพของวิธีเมทิลเลชันสเปคซิฟิคทริพเพลทไพร์มพีซีอาร์ (เอ็มเอสทีพีพีซีอาร์) โดยพิจารณาการขยายเพิ่มจำนวนซ้ำของซีจีจีและการเกิดเมทิลเลชันของยีนเอฟเอ็มอาร์วันในตัวอย่างชายและหญิงจากโรงพยาบาลสงขลานครินทร์ ขั้นตอนเริ่มจากการดัดแปลงโครงสร้างดีเอ็นเอจากจีโนมด้วยโซเดียมไบซัลไฟต์ จากนั้นเพิ่มจำนวนดีเอ็นเอที่ถูกดัดแปลงด้วยวิธีเอ็มเอสทีพีพีซีอาร์ แล้ววิเคราะห์ผลด้วยเทคนิคอิเล็กโตรโฟรีซิส จากการศึกษาพบว่า ตัวอย่างที่ทดสอบทั้งหมดให้ผลการทดสอบถูกต้องและสอดคล้องกับผลจากการวิเคราะห์ด้วยวิธีพีซีอาร์และ/หรือการวิเคราะห์เซาเทิร์นบลอท ดังนั้นวิธีเอ็มเอสทีพีพีซีอาร์นี้อาจเป็นอีกทางเลือกหนึ่งสำหรับใช้ตรวจวินิจฉัยกลุ่มอาการโครโมโซมเอกซ์เปราะในห้องปฏิบัติการของโรงพยาบาลสงขลานครินทร์ซึ่งสามารถลดความต้องการในการตรวจวิเคราะห์ด้วยเซาเทิร์นบลอทได้

Keywords: Fragile X syndrome, Methylation-specific triplet-primed PCR, Southern blot analysis
คำสำคัญ: กลุ่มอาการโครโมโซมเอกซ์เปราะ เมทิลเลชันสเปคซิฟิคทริพเพลทไพร์มพีซีอาร์ การวิเคราะห์เซาเทิร์นบลอท

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Introduction

Fragile X syndrome (FXS) is the most common X-linked disorder that causes mental impairment and autism. It is characterized by intellectual disability (ID), hyperactivity, language delay, social and behavioral issues, and other physical impairments, affecting estimated about 1 in 7,000 males and about 1 in 11,000 females (Hunter et al., 2014). The disorder is caused by mutation of the fragile X mental retardation-1 (*FMR1*) gene and is related to hyperexpansion and hypermethylation of a Cytosine-Guanine-Guanine (CGG) triplet-repeat sequence at the 5'untranslated region (5'UTR) of the *FMR1* gene (Biancalana et al., 2015). The *FMR1* alleles can be divided into 4 groups depending on the *FMR1* CGG repeat numbers; normal (NL) alleles: 5 to 44 repeats, intermediate or gray zone (IM or GZ) alleles: 45 to 54 repeats, premutation (PM) alleles: 55 to 200 repeats, and full mutation (FM) alleles: >200 repeats. Individuals with more than 200 CGG repeats typically present with clinical symptoms of FXS (Monaghan et al., 2013).

Nowadays, the molecular diagnostic testing for FXS is usually based on CGG repeat sizing and detecting of the methylation status of the *FMR1* gene. The most commonly used techniques for FXS diagnosis are a combination of PCR for determination of CGG repeat sizes up to 100-150 repeats (Filipovic-Sadic et al., 2010) and Southern blot analysis for detection of larger sizes and methylation status of the *FMR1* gene. However, this combination is laborious, requires a large amount of DNA, takes a long time, and is not suitable for testing in large volumes. As a result, various FXS diagnostic techniques have been developed to improve these limitations (Filipovic-Sadic et al., 2010; Lyon et al., 2010; Rajan-Babu, Chong, 2016). The challenge of FXS diagnosis is detection of the samples containing premutation or full mutation alleles, especially in females because of the high GC content and preferential amplification of smaller alleles, leading to nonamplification of larger alleles. Furthermore, at least 25% of female samples are homozygous, which cannot be separated from heterozygous upper premutation or full mutation carrier females by conventional PCR (Juusola et al., 2012). The current FXS diagnosis in the routine laboratory at Songklanagarind Hospital uses the direct triplet-primed PCR (TP-PCR) method for resolving the apparent homozygosity in female samples. Nevertheless, this method is difficult to discern between premutation and full mutation and cannot identify methylation status of the *FMR1* allele. Thus, Southern blot analysis is still to be performed along with conventional PCR, direct TP-PCR, and methylation-specific PCR (MS-PCR) methods.

Methylation-specific triplet-primed PCR (msTP-PCR) is an attractive method that can overcome the limitations of TP-PCR by using bisulfite-treated DNA as the template for PCR amplification. The modification of genomic DNA via bisulfite treatment can differentiate unmethylated and methylated templates based on methylation status. For msTP-PCR amplification of each modified DNA template (unmethylated or methylated alleles), three oligonucleotide primers are specifically used: (a) a fluorescent locus-specific primer that binds to downstream or upstream of the CGG repeat sequences, (b) a triplet-primed (TP) primer that hybridizes randomly within the CGG repeat regions giving a mixture of different sizes of PCR products, and these yields are further amplified to boost the fluorescent signal by (c) a tail primer (Warner et al., 1996). Therefore, this msTP-PCR method designs not only for detection of trinucleotide repeat expansion but also identification of

methylation status and approximately CGG repeat size. There is little utilization of the msTP-PCR method in the routine laboratory, which this method could reduce the need for Southern blot analysis.

Objective of the study

To validate performance of the modified msTP-PCR method for FXS diagnosis in the routine laboratory using DNA samples covering all ranges (genotypes) of the *FMR1* alleles received from Songklanagarind Hospital.

Materials and methods

Study design and clinical DNA samples

This study was a preliminary study that was employed with 35 leftover specimens taken from individuals who had clinical symptoms of FXS or had family history of FXS referred to FXS testing at Songklanagarind Hospital. These DNA samples were already processed for their CGG repeats and methylation status to classify the *FMR1* genotype using conventional PCR and/or Southern blot analysis. This study was approved by the Ethics Committee of the Faculty of Medicine, Prince of Songkla University (REC.62-018-5-2).

Bisulfite modification of genomic DNA

All genomic DNA samples were modified with sodium bisulfite using the EZ DNA Methylation-Gold Kit (Zymo Research, USA) before its use in the modified msTP-PCR method. The bisulfite treatment protocol was carried out according to a previous study with some modifications (Rajan-Babu et al., 2015). The bisulfite modification of the DNA changes unmethylated cytosine residues to uracil, whereas leaves methylated cytosine residues unaffected.

Methylation-specific triplet-primed PCR (msTP-PCR: uTP-PCR and mTP-PCR)

The bisulfite-treated DNA samples were amplified with two sets of primers to detect CGG repeat expansion and methylation status of the *FMR1* gene. The msTP-PCR was divided into unmethylated triplet-primed PCR (uTP-PCR) and methylated triplet-primed PCR (mTP-PCR) reactions that are specific with unmethylated and methylated *FMR1* alleles, respectively. Primers Fam-uFMR1-R (Fam-labeled reverse primer) (5'TGTTTTTGAGAGGTGGGTTGTGGGTGTTT3'), uFMR1-TPF (unlabeled triplet-primed forward primer) (5'ATTGGGTCCAGTCGCTTTAGG$(CAA)_9$3'), and uTail (5'ATTGGGTCCAGTCGCTTTAGG3') were used for uTP-PCR as well as primers VIC-mFMR1-R (VIC-labeled reverse primer) (5'CGTTTTCGAGAGGTGGGTTGCGGGCGTTC3'), mFMR1-TPF (unlabeled triplet-primed forward primer) (5'ATTCCATCCCAGTTTGTCAGC$(CGA)_8$3'), and mTail (5'ATTCCATCCCAGTTTGTCAGC3') were used for mTP-PCR. These primer sequences are identical to the primers of the previous publication (Rajan-Babu et al., 2015). The Fam-uFMR1-R and VIC-mFMR1-R primers are fluorescently tagged locus-specific primers, which bind to the downstream of the CGG repeats. For random annealing within the CGG repeats,

uFMR1-TPF and mFMR1-TPF were performed. Furthermore, uTail and mTail primers correspond to the sequence of its forward primer that can improve the signals of the fragments amplified.

PCR master mixes of uTP-PCR were carried out in a 50 µl containing 0.2 mM dNTPs, 2.5 U HotStarTaq™ DNA polymerase (Qiagen, Germany), 1X supplied PCR buffer (Qiagen, Germany), 1.5X Q-solution (Qiagen, Germany), 2.0 mM $MgCl_2$, 6% dimethyl sulfoxide (DMSO), and 250 ng of bisulfite-treated DNA. Primer concentrations of Fam-uFMR1-R, uFMR1-TPF, and uTail primers for uTP-PCR were 0.6, 0.6, and 0.006 µM, respectively. For PCR reaction of mTP-PCR was similar to uTP-PCR, except that 0.25 mM dNTPs were used instead. The primer concentrations of VIC-mFMR1-R, mFMR1-TPF, and mTail primers for mTP-PCR were 0.4, 0.4, and 0.0004 µM, respectively. Thermal cycling achieved with an ABI 9700 thermal cycler, was started by an initial denaturation at 95°C for 15 min, followed by 40 cycles of 98°C for 1.5 min, 60°C for 1 min, and 72°C for 3 min, followed by a final extension at 72°C for 10 min.

Capillary electrophoresis analysis

After msTP-PCR amplification, msTP-PCR products (uTP-PCR and mTP-PCR products) were analyzed with the 3500 Genetic Analyzer (Applied Biosystems). Briefly, 2 µl aliquot of each PCR product from each sample was mixed with 0.5 µl of GeneScan™ 600 LIZ® Size Standard v2.0 (Applied Biosystems, USA) and 9 µl of HiDi formamide (Applied Biosystems, USA), heat-denatured at 95°C for 5 min, followed by cooling at 4°C for 5 min before transferring to capillary electrophoresis (CE) instrument (CE, 50 cm, POP-7, 5 sec 1 kV injection, 5800 sec 10 kV run). When the CGG repeat expansions were detected in samples, those samples were subsequently subjected to a second CE running (CE, 50 cm, POP-7, 15 sec 5 kV injection, 3000 sec 10 kV run)

Data analysis and result interpretation

GeneMapper® software 5 (Applied Biosystems) was used for detecting electropherograms. The CGG repeat expansion and methylation status of the *FMR1* gene were defined by analyzing the electropherograms in the blue fluorescent dye (*Fam*) channel for unmethylated *FMR1* alleles and the green fluorescent dye (*VIC*) channel for methylated *FMR1* alleles. Each electropherogram was classified into one of three possible ranges: (a) 74 to 221 base pairs (bps) or 5 to 54 CGG repeats (normal and intermediate), (b) 222 to 419 bps or 55 to 120 CGG repeats (lower premutation), and (c) >419 bps or >120 CGG repeats (upper premutation and full mutation). The final peak of each sample was determined depending on the morphology of the peak and/or signal height. The *FMR1* genotype classification, according to the American College of Medical Genetics and Genomics guidelines (Monaghan et al., 2013).

The workflow of this study was shown in Figure 1. The process started by bisulfite modification of DNA samples. The treated DNA was then amplified using the modified msTP-PCR method and analyzed by capillary electrophoresis.

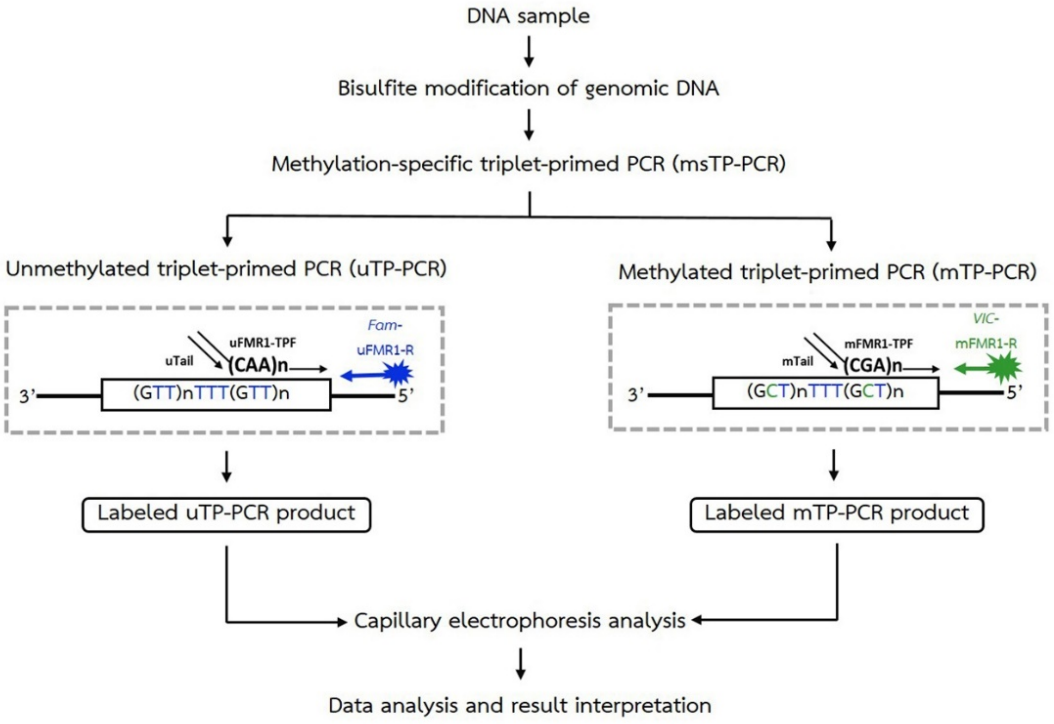

Figure 1 The workflow for performing with DNA samples using the modified msTP-PCR method.

Results

This study was achieved to validate performance of the modified msTP-PCR method using 35 known DNA samples that were previously characterized for their CGG repeat sizes and methylation status of the *FMR1* gene representing normal, intermediate, premutation, and full mutation of both male and female samples as well as mosaicism samples. The example electropherogram results of both males and females were shown in Figure 2. Each fluorescent msTP-PCR channel was divided into sub-channels according to the result interpretation. Based on the CGG expansion profiles and the methylation status, the modified msTP-PCR method can classify the categories of the *FMR1* alleles and provides the AGG interruptions for the respective non-expanded allele and expanded allele samples of both males and females.

To prove the efficiency of the modified msTP-PCR method, the optimized PCR conditions were tested in known samples with variable *FMR1* CGG repeat classifications.

Validation on female samples

Females have two X-chromosomes, one of which is methylated via the random X-inactivation, thus two *FMR1* alleles with different CGG repeat sizes were expected to generate PCR products for both uTP-PCR and mTP-PCR channels. In the heterozygous normal female: M59-61 (Fig. 2A), the PCR products appeared in both uTP-PCR and mTP-PCR channels. The PCR product from each channel generated three discrete clusters that were separated by two clear zones, representing a common normal pattern. These clear zones indicated the AGG interruption in the *FMR1* allele. In a female sample with premutation allele: M60-85 (Fig. 2C), the continuous series of fluorescent peaks for the expanded allele and discrete clusters for the normal allele were

overlapped in both uTP-PCR and mTP-PCR channels. Moreover, the modified msTP-PCR method can distinguish homozygous normal female: M59-52 (Fig. 2B) who carries the same CGG repeat sizes of two *FMR1* alleles from heterozygous full mutation carrier female: F20-6 (Fig. 2D) who carries one normal allele (36 CGG repeats) and one full mutation allele (>200 CGG repeats). The result of full mutation female showed the CGG expansion in mTP-PCR channel, indicated the methylated full mutation allele. These represented that the modified msTP-PCR method can resolve the ambiguity of zygosities in female samples.

Validation on male samples

Normal males contain only one X-chromosome that is always unmethylated. Therefore, only PCR product was yielded in uTP-PCR channel. Likewise, the premutation alleles in males are also unmethylated and should be amplified from uTP-PCR reaction only. For male samples with normal: M63-274 (Fig. 2E) and premutation: F4-7 (Fig. 2F) genotypes, the electropherograms correctly showed a normal pattern for normal male and a continuous series of fluorescent peaks for premutation male in uTP-PCR channel, respectively. For male samples, if no PCR product is formed in uTP-PCR channel but presents an interrupted series of fluorescent peaks in mTP-PCR channel, it indicates methylated full mutation allele that can interpret as full mutation male: M62-25 (Fig. 2G). One important point for FXS testing is samples with CGG repeat size mosaicism in which individuals present with different CGG repeat allele sizes that is common among individuals with FXS and should be identified by Southern blot analysis. The modified msTP-PCR method in this study can identify a sample with mosaicism for *FMR1* premutation and full mutation alleles: M57-38 (Fig. 2H).

After PCR was performed under optimal condition, we further tested this method under the same condition in other 35 known DNA samples that included 12 non-expanded allele and 23 expanded allele samples to validate the accuracy of this method, and compared the results between the modified msTP-PCR method and the standard diagnostic methods.

Validation on non-expanded allele samples

A total of 12 non-expanded allele samples, 9 normal and 3 intermediate samples of both males and females, was included in this study (Table 1). According to the CGG expansion profiles and the methylation status results of the modified msTP-PCR method, all of the modified msTP-PCR results in non-expanded allele samples corresponded to the results from the standard diagnostic methods (combination of conventional PCR and/or Southern blot analysis).

Validation on expanded allele samples

The 23 expanded allele samples consisted of 9 premutation, 9 full mutation, and 5 mosaicism samples of both males and females (Table 2). The modified msTP-PCR method identified and generated unique patterns for all types of expanded allele samples depending on the CGG expansion and methylation status, which corresponded to the standard diagnostic methods.

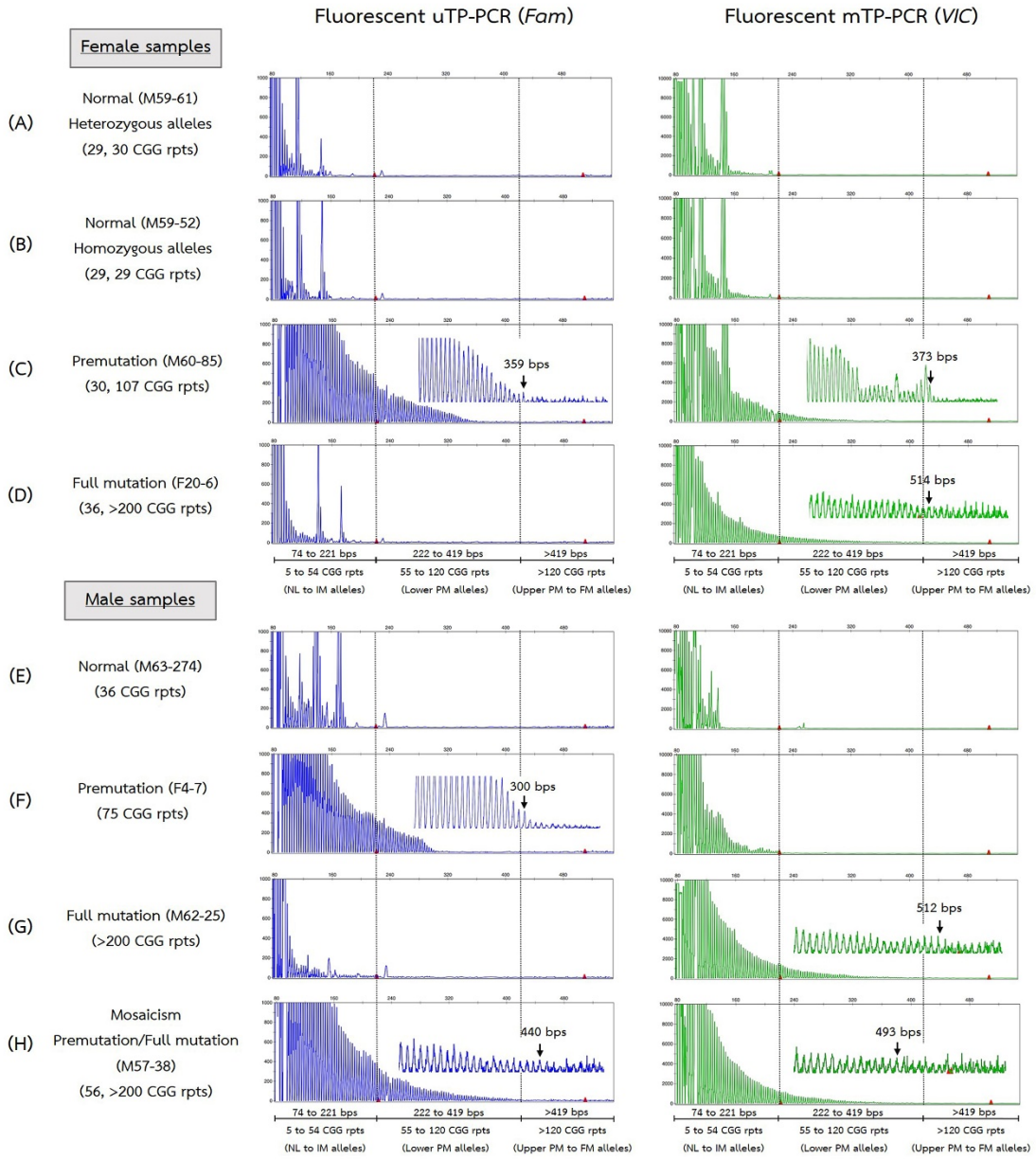

Figure 2 The methylation-specific triplet-primed PCR (msTP-PCR) GeneScan profiles of individuals with normal, premutation, full mutation, and mosaicism of the *FMR1* gene. The electropherograms present in the fluorescent uTP-PCR and the fluorescent mTP-PCR channels are zoomed to 1,000 and 10,000 relative fluorescence units (RFU), respectively to illustrate CGG expansion and methylation status of unmethylated and methylated *FMR1* alleles. The arrow indicates the final peak of each *FMR1* allele. The msTP-PCR GeneScan profiles of females with heterozygous normal, homozygous normal, premutation, and full mutation alleles are shown as (A), (B), (C), and (D), respectively. The msTP-PCR GeneScan profiles of males with normal, premutation, full mutation, and mosaicism for premutation and full mutation alleles are shown as (E), (F), (G), and (H), respectively. (NL: Normal; IM: Intermediate; PM: Premutation; FM: Full mutation; rpts: Repeats; bps: Base pairs)

Table 1 Assay validation on non-expanded allele samples of males and females

Sample ID/sex	Conventional PCR and/or Southern blot analysis (Standard diagnostic methods)		Modified msTP-PCR (This study)		
	CGG repeat size	CGG repeat classification	uTP-PCR: CGG repeat expansion and methylation status	mTP-PCR: CGG repeat expansion and methylation status	CGG repeat classification
M57-21/F	29, 29	Normal	Not expanded in normal range (Unmet)	Not expanded in normal range (Met)	Normal
M57-31/F	29, 29	Normal	Not expanded in normal range (Unmet)	Not expanded in normal range (Met)	Normal
M59-46/F	23, 39	Normal	Not expanded in normal range (Unmet)	Not expanded in normal range (Met)	Normal
M59-52/F	29, 29	Normal	Not expanded in normal range (Unmet)	Not expanded in normal range (Met)	Normal
M59-61/F	29, 30	Normal	Not expanded in normal range (Unmet)	Not expanded in normal range (Met)	Normal
M63-59/M	30	Normal	Not expanded in normal range (Unmet)	No PCR product	Normal
M63-113/M	29	Normal	Not expanded in normal range (Unmet)	No PCR product	Normal
M63-184/M	29	Normal	Not expanded in normal range (Unmet)	No PCR product	Normal
M63-274/M	36	Normal	Not expanded in normal range (Unmet)	No PCR product	Normal
AR54-2/F	30, 53	Intermediate	Not expanded in intermediate range (Unmet)	Not expanded in intermediate range (Met)	Intermediate
AR54-3/M	53	Intermediate	Not expanded in intermediate range (Unmet)	No PCR product	Intermediate
M57-41/M	47	Intermediate	Not expanded in intermediate range (Unmet)	No PCR product	Intermediate

Not expanded allele: <55 CGG repeat sizes; F: Female; M: Male; Unmet: Unmethylated; Met: Methylated

Table 2 Assay validation on expanded allele samples of males and females

Sample ID/sex	Conventional PCR and/or Southern blot analysis (Standard diagnostic methods)		Modified msTP-PCR (This study)		
	CGG repeat size	CGG repeat classification	uTP-PCR: CGG repeat expansion and methylation status	mTP-PCR: CGG repeat expansion and methylation status	CGG repeat classification
F3-2/F	29, 110	Premutation	Not expanded in normal range (Unmet) and expanded in lower premutation range (Unmet)	Not expanded in normal range (Met) and expanded in lower premutation range (Met)	Premutation
F10-5/F	29, 85	Premutation	Not expanded in normal range (Unmet) and expanded in lower premutation range (Unmet)	Not expanded in normal range (Met) and expanded in lower premutation range (Met)	Premutation
F10-6/F	29, 85	Premutation	Not expanded in normal range (Unmet) and expanded in lower premutation range (Unmet)	Not expanded in normal range (Met) and expanded in lower premutation range (Met)	Premutation
F21-2/F	30, 70	Premutation	Not expanded in normal range (Unmet) and expanded in lower premutation range (Unmet)	Not expanded in normal range (Met) and expanded in lower premutation range (Met)	Premutation
M60-85/F	30, 107	Premutation	Not expanded in normal range (Unmet) and expanded in lower premutation range (Unmet)	Not expanded in normal range (Met) and expanded in lower premutation range (Met)	Premutation
M61-79/F	30, 79	Premutation	Not expanded in normal range (Unmet) and expanded in lower premutation range (Unmet)	Not expanded in normal range (Met) and expanded in lower premutation range (Met)	Premutation
NC58-18/F	36, 95	Premutation	Not expanded in normal range (Unmet) and expanded in lower premutation range (Unmet)	Not expanded in normal range (Met) and expanded in lower premutation range (Met)	Premutation
F4-7/M	75	Premutation	Expanded in lower premutation range (Unmet)	No PCR product	Premutation
F31-6/M	80	Premutation	Expanded in lower premutation range (Unmet)	No PCR product	Premutation

Table 2 Assay validation on expanded allele samples of males and females (Cont.)

Sample ID/sex	Conventional PCR and/or Southern blot analysis (Standard diagnostic methods)		Modified msTP-PCR (This study)		
	CGG repeat size	CGG repeat classification	uTP-PCR CGG repeat expansion and methylation status	mTP-PCR CGG repeat expansion and methylation status	CGG repeat classification
F3-10/F	29, >200	Full mutation	Not expanded in normal range (Unmet)	Not expanded in normal range (Met) and expanded in upper premutation and full mutation range (Met)	Full mutation
F20-6/F	36, >200	Full mutation	Not expanded in normal range (Unmet)	Not expanded in normal range (Met) and expanded in upper premutation and full mutation range (Met)	Full mutation
F11-3/M	>200	Full mutation	No PCR product	Expanded in upper premutation and full mutation range (Met)	Full mutation
F35-3/M	>200	Full mutation	No PCR product	Expanded in upper premutation and full mutation range (Met)	Full mutation
F36-3/M	>200	Full mutation	No PCR product	Expanded in upper premutation and full mutation range (Met)	Full mutation
M58-2/M	>200	Full mutation	No PCR product	Expanded in upper premutation and full mutation range (Met)	Full mutation
M62-16/M	>200	Full mutation	No PCR product	Expanded in upper premutation and full mutation range (Met)	Full mutation
M62-22/M	>200	Full mutation	No PCR product	Expanded in upper premutation and full mutation range (Met)	Full mutation
M62-25/M	>200	Full mutation	No PCR product	Expanded in upper premutation and full mutation range (Met)	Full mutation
CR4-3/M	29, 85,>200	Mosaicism for Normal/ Premutation/ Full mutation	Not expanded in normal range (Unmet) and expanded in lower premutation range (Unmet)	Expanded in upper premutation and full mutation range (Met)	Mosaicism for Normal/ Premutation/ Full mutation
CR68-3M	30, 114, >200	Mosaicism for Normal/ Premutation/ Full mutation	Not expanded in normal range (Unmet) and expanded in lower premutation range (Unmet)	Expanded in upper premutation and full mutation range (Met)	Mosaicism for Normal/ Premutation/ Full mutation
M57-38/M	56, >200	Mosaicism for Premutation/ Full mutation	Expanded in upper premutation and full mutation range (Unmet)	Expanded in upper premutation and full mutation range (Met)	Mosaicism for Premutation/ Full mutation
M57-96/M	150, >200	Mosaicism for Premutation/ Full mutation	Expanded in upper premutation and full mutation range (Unmet)	Expanded in upper premutation and full mutation range (Met)	Mosaicism for Premutation/ Full mutation
M60-61/M	159, >200	Mosaicism for Premutation/ Full mutation	Expanded in upper premutation and full mutation range (Unmet)	Expanded in upper premutation and full mutation range (Met)	Mosaicism for Premutation/ Full mutation

Not expanded allele: <55 CGG repeat sizes; Expanded allele: ≥55 CGG repeat sizes; F: Female; M: Male; Unmet: Unmethylated; Met: Methylated

According to the modified msTP-PCR results of 35 tested samples, all of the modified msTP-PCR results corresponded to the results from the standard diagnostic methods. Thus, the sensitivity and specificity of the modified msTP-PCR for identification of *FMR1* non-expanded and expanded alleles in variable samples in this study were 100%.

Discussion and Conclusions

The concept of the modified msTP-PCR method is based on TP-PCR method that was first described by Warner et al. (1996). They have designed this method to detect CAG expansion of myotonic dystrophy. Primer designing of TP-PCR assay is unique and suitable for identifying trinucleotide repeat expansions, including FXS, spinocerebellar ataxia (SCA), Huntington's disease (HD), and Kennedy's disease (KD) (Aydin et al., 2018; Chheda et al., 2018). To assess methylation status and the expansion of trinucleotide repeats, msTP-PCR has been developed. For the current FXS diagnosis, the msTP-PCR method is increasingly being used because this method provides not only identification of the CGG expansion, methylation status, and approximately CGG repeat size but also offers the AGG interruption information, which affects the stability of the *FMR1* gene and decreases the risk of CGG expansion during transmission (Villate et al., 2020). In this study, we described performance of the modified msTP-PCR method with clinical DNA samples, the CGG expansions were detected by a combination of the three primers performing to amplify the triplet repeat expansions and produced msTP-PCR products of various sizes that can be visualized as stutters of fluorescent peaks. The identification of the methylation status relies on the different bisulfite modification of genomic DNA of unmethylated and methylated *FMR1* alleles and subsequent PCR amplification. We found non-specificity as minimal peaks in mTP-PCR channel of some normal male (M63-274) and premutation male (F4-7) samples that were also reported in the previous study (Rajan-Babu et al., 2015). This issue is likely caused by overamplification because of excessive template or components in the PCR master mix; nonetheless, it did not affect interpretation. In mosaicism for *FMR1* premutation and full mutation alleles (M57-38), the expected electropherogram in uTP-PCR channel of this sample should not expand more than 419 base pairs or 120 repeats because his premutation allele carries 56 CGG repeats; however, the true result showed expansion of unmethylated *FMR1* allele in uTP-PCR channel at 440 base pairs. This mosaicism sample may consist of more than one size of unmethylated *FMR1* alleles but too large to be amplified by conventional PCR and might be a very small amount of its population, making it impossible to detect by Southern bot analysis. Although the expected amplicon sizes of full mutation alleles are greater than 200 CGG repeats, all tested samples with full mutation alleles presented at more than 120 CGG repeats or 419 base pairs using the modified msTP-PCR method because of the limitation of the PCR reagents and analytic tools in our laboratory. However, this limitation can be improved by interpretation of msTP-PCR based on both uTP-PCR and mTP-PCR.

A previous publication by Rajan-Babu et al. (2015) suggested that the PCR amplification can carry out in a single tube; however, in our experience this was difficult for performing in the sample containing more than one *FMR1* alleles, especially in female and mosaicism samples due to competition of PCR amplification in limited master mix components, making low fluorescent intensity level of larger alleles of PCR products.

Overall, we ensured that the modified msTP-PCR in this study can accurately classify all *FMR1* genotypes. The msTP-PCR results were consistent with the results of the already-known genotypes of leftover specimens received from Songklanagarind Hospital using conventional PCR and/or Southern blot analysis as the standard methods. Additionally, we compared the modified msTP-PCR method with the methods use in the routine laboratory at Songklanagarind Hospital in Table 3, which found that each method has different advantages and disadvantages. However, the gold standard method for FXS diagnosis is still Southern blot analysis.

We proposed that the modified msTP-PCR method is effective, less time consuming, requires small amounts of DNA, and inexpensive. This method can be useful as an alternative assay for FXS diagnosis in the routine laboratory testing. Nevertheless, it should be noted that this preliminary study should be further performed in greater volume of samples as well as calculate statistical parameters to evaluate the new method.

Table 3 Comparison of the modified msTP-PCR method with the methods in the routine laboratory at Songklanagarind Hospital for FXS diagnosis

Comparison lists	Modified msTP-PCR	Direct TP-PCR	Southern blot analysis (Gold standard method)
Time required	~ 2 days	~ 2 days	~ 5 days
DNA quantity (micrograms)	0.1-0.5	0.1-0.5	8.0-10.0
CGG repeat sizing	Approximately	Approximately	Approximately
CGG expansion detection	Yes (male, female)	Yes (male, female)	Yes (male, female)
Methylation status	Yes (male, female)	No	Yes (male, female)
Reagents cost	Inexpensive (~ 350 Baht/reaction)	Inexpensive (~ 250 Baht/reaction)	Expensive (~ 2,000 Baht/reaction)

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