# 3. Results and discussion

## Results

### 3.1 PCR for CGG repeats

DNA analysis provides a reliable diagnosis of FXS. The PCR is mainly applied to determine the number of the triplet repeats. Fig. 6 shows an example of PCR for CGG repeats.

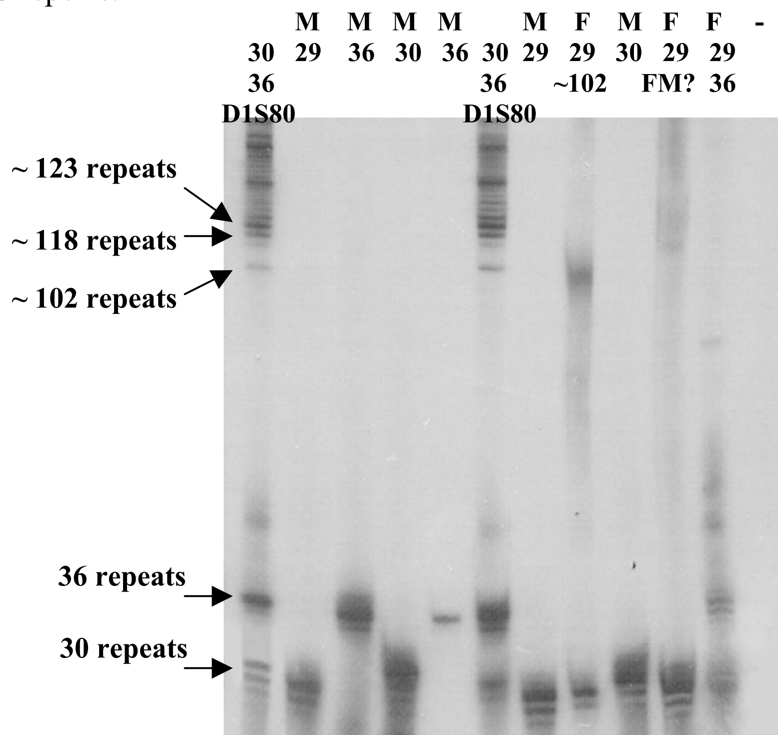


**Fig. 6 An example of results from PCR for CGG repeats.** M is a marker, indicating known CGG repeats (30 and 36 repeats) and the D1S80 marker. From the left, lane 2 and 7 are normal males with 29 CGG repeats. Lane 3 and 5 are normal males with 36 CGG repeats. Lane 4 and 9 are normal males 30 CGG repeats. Lane 8 is a premutation female with 29 and ~ 102 CGG repeats. Lane 10 is a suspected full mutation (FM) female with 29 and variable numbers of CGG repeats (smear). Lane 11 is a normal female with 29 and 36 repeats. Lane 12 is a negative control. M = male and F = female.

Although PCR is highly sensitive for allele sizes with normal and low premutation ranges, it may fail to detect the allele sizes in the upper premutation and full mutation ranges due to a high GC content of the repeat region. Thus, we could not distinguish whether amplification failure or full mutation alleles were present. Also, it was unable to distinguish homozygous females from full mutation females. Individuals with full mutation and upper premutation ranges may appear as smear as shown in Fig. 6, lane 8 and 10.

### 3.2 Southern blot analysis

Southern blot hybridization is a further test for questionable cases or inconclusive PCR results. Genomic DNA was digested by using a methylation sensitive restriction enzyme (*Eag*I) and a methylation insensitive restriction enzyme (*Eco*RI), subsequently hybridized with a specific probe (StB12.3). The active X chromosome is always cut by *Eag*I, resulting in 2.8 Kb fragment. The inactive X chromosome and the methylated *FMR*1 gene in full mutations are not cut by *Eag*I, resulting in 5.2 Kb and larger than 5.2 Kb fragments, respectively. Following this method, methylation status and approximate size of CGG repeats is obtained (Fig.7 and Fig. 8).

**Determination of the CGG size from Southern blot analysis**

The approximate number of CGG repeats can be calculated from Southern blot analysis by using a semi-log graph (Fig. 9 and Fig. 10). Following these steps:

1. Determine the axes. The X axis is a distance from a reference line (cm) and the Y axis is a size of 2-log marker (Kb). The reference line is an imaginary line between 2 Kb bands of 2-log marker at each side of the blot.

2. Generate a standard curve by measurement of distance between each band of 2-log markers and a reference line.

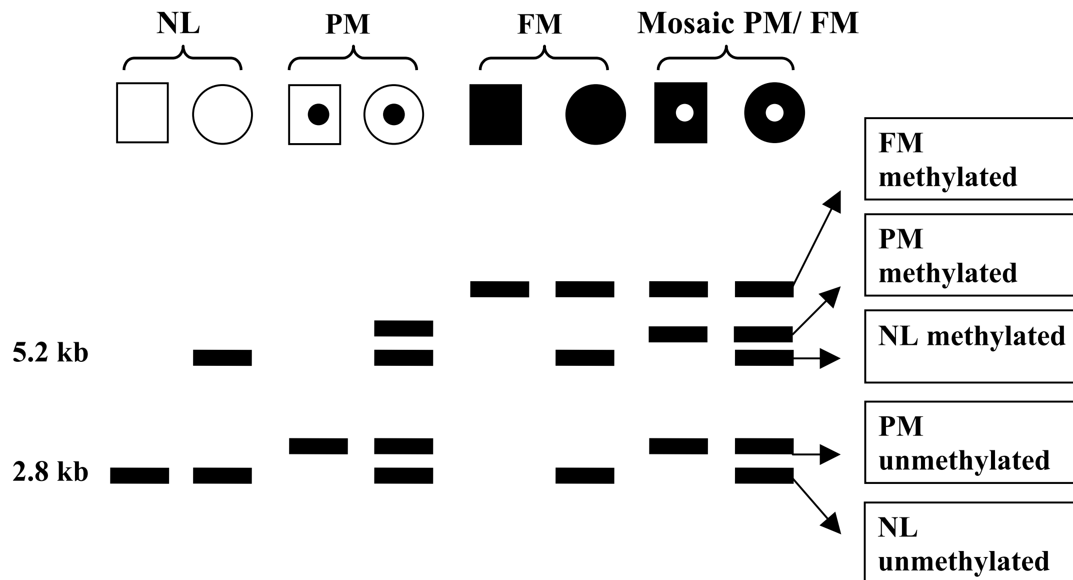


**Fig. 7 Diagrammatic presentation of the results from a typical Southern blot analysis.** A normal male has one active X chromosome, thus only one band of 2.8 Kb is detected. A normal female has one active X chromosome and one inactive X chromosome, given 2.8 Kb and 5.2 Kb bands, respectively. When the number of CGG repeats is higher than normal, the length of the digested product is extended. In a premutation male, a band with larger than 2.8 Kb is detected. In a premutation female, a 2.8 Kb and a larger than 2.8 Kb bands are usually detected as a result from digestion of the active X chromosome, and a 5.2 kb and a larger than 5.2 Kb bands are also detected as a result from digestion of the inactive X chromosome. In a full mutation male, there are over 200 CGG repeats, resulting in methylation of the *FMR*1 gene and given band(s) with larger than 5.2 Kb. In a full mutation female, 2.8 Kb, 5.2 Kb and band(s) with larger than 5.2 Kb are detected. The pattern of digested product observed in mosaic premutation and full mutation (mosaic PM/FM) is composed of both patterns of premutation and full mutation. NL = normal, PM = premutation and FM = full mutation.

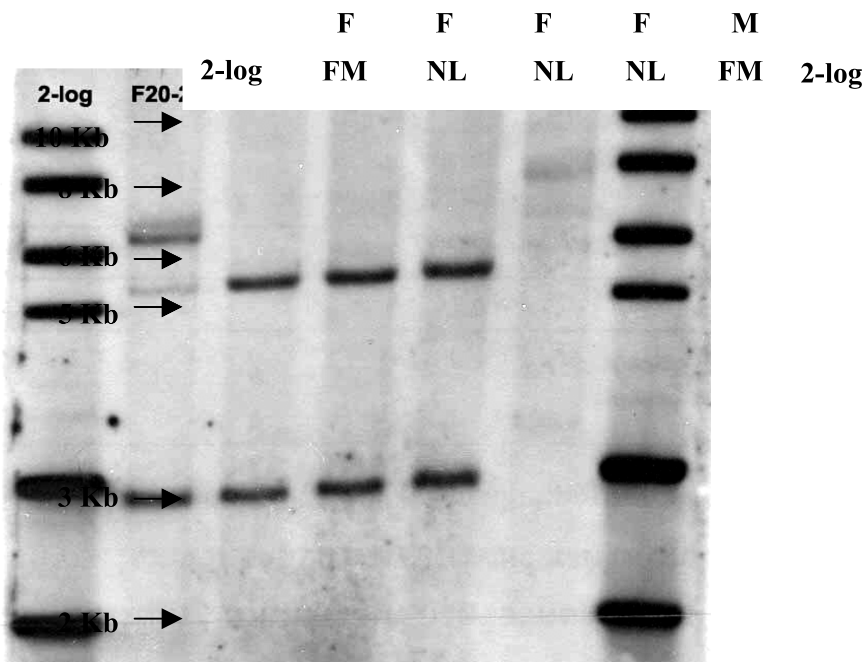


**Fig. 8 An example of results from Southern blot analysis**. From the left, lane 1 and 7 are 2-log DNA markers. Lane 2 is a full mutation female with one band of 2.8 Kb, one band of 5.2 Kb and one band grater than 5.2 Kb. Lane 3, 4 and 5 are normal females with 2.8 kb and 5.2 kb bands. Lane 6 is a full mutation male with multiple bands larger than 5.2 Kb. M = male, F = female, NL = normal, PM = premutation and FM = full mutation.

3. Plot "A" on a standard curve, where A is the distance between a band of sample DNA and a reference line (cm).

4. Draw a line from "A" to the Y axis, given "B" (Kb).

5. Subtract"B" with 5.2 Kb, given "C" (Kb).

6. Calculate the CGG repeats using the formula:

$$\frac{(C \times 1000)}{3} + 30 \text{ repeats}$$

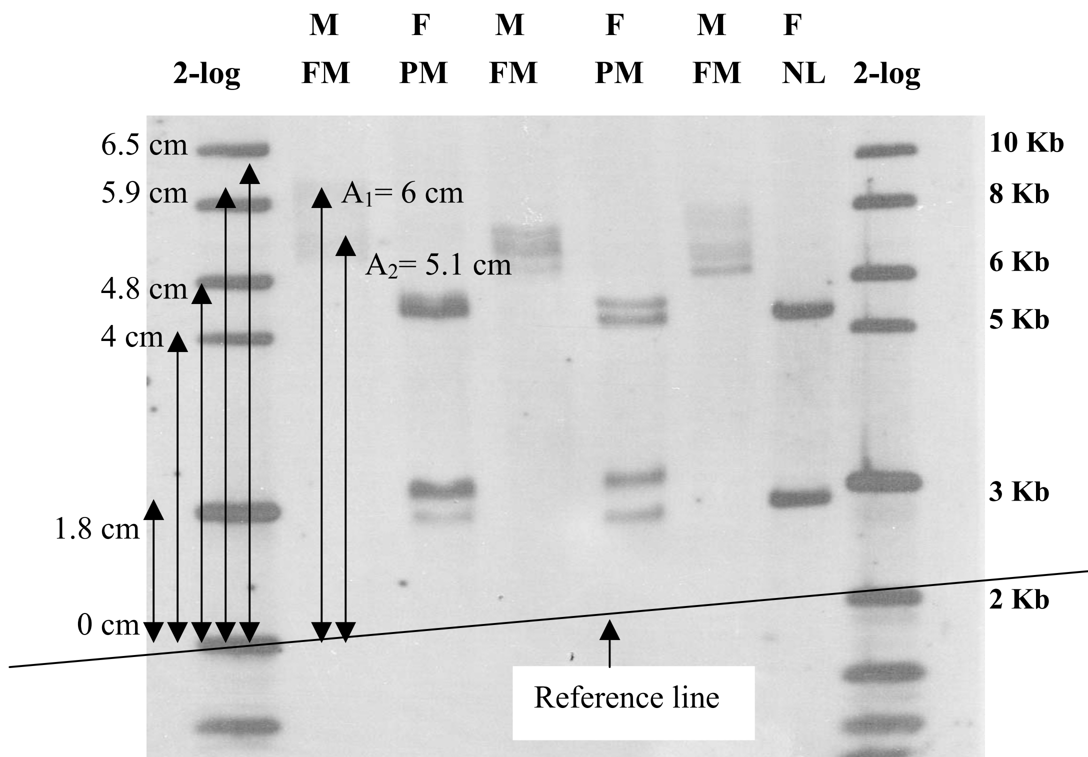


**Fig. 9 An example of CGG repeats calculation from Southern blot analysis.** An individual in lane 2 is a full mutation male, comprised of two bands. $A_1$ is a distance between the first band and a reference line (6 cm) and $A_2$ is a distance between the second band and a reference line (5.1 cm). A standard curve is plotted on a semi-log graph using a distance between each band of 2-log markers and a reference line. $A_1$ and $A_2$ are plotted on a standard curve. Draw a line from $A_1$ and $A_2$ to the Y axis. $B_1$ and $B_2$ are approximate 7 Kb and 8.9 Kb, respectively (Fig. 10).

$C_1$ = 7-5.2 Kb = 1.8 Kb

$C_2$ = 8.9-5.2 Kb = 3.7 Kb

Therefore, this full mutation individual has approximate CGG repeats;

$$\left(\frac{1.8 \times 1000}{3}\right) + 30 = 630 \text{ repeats}$$

$$\left(\frac{3.7 \times 1000}{3}\right) + 30 = 1{,}263 \text{ repeats}$$

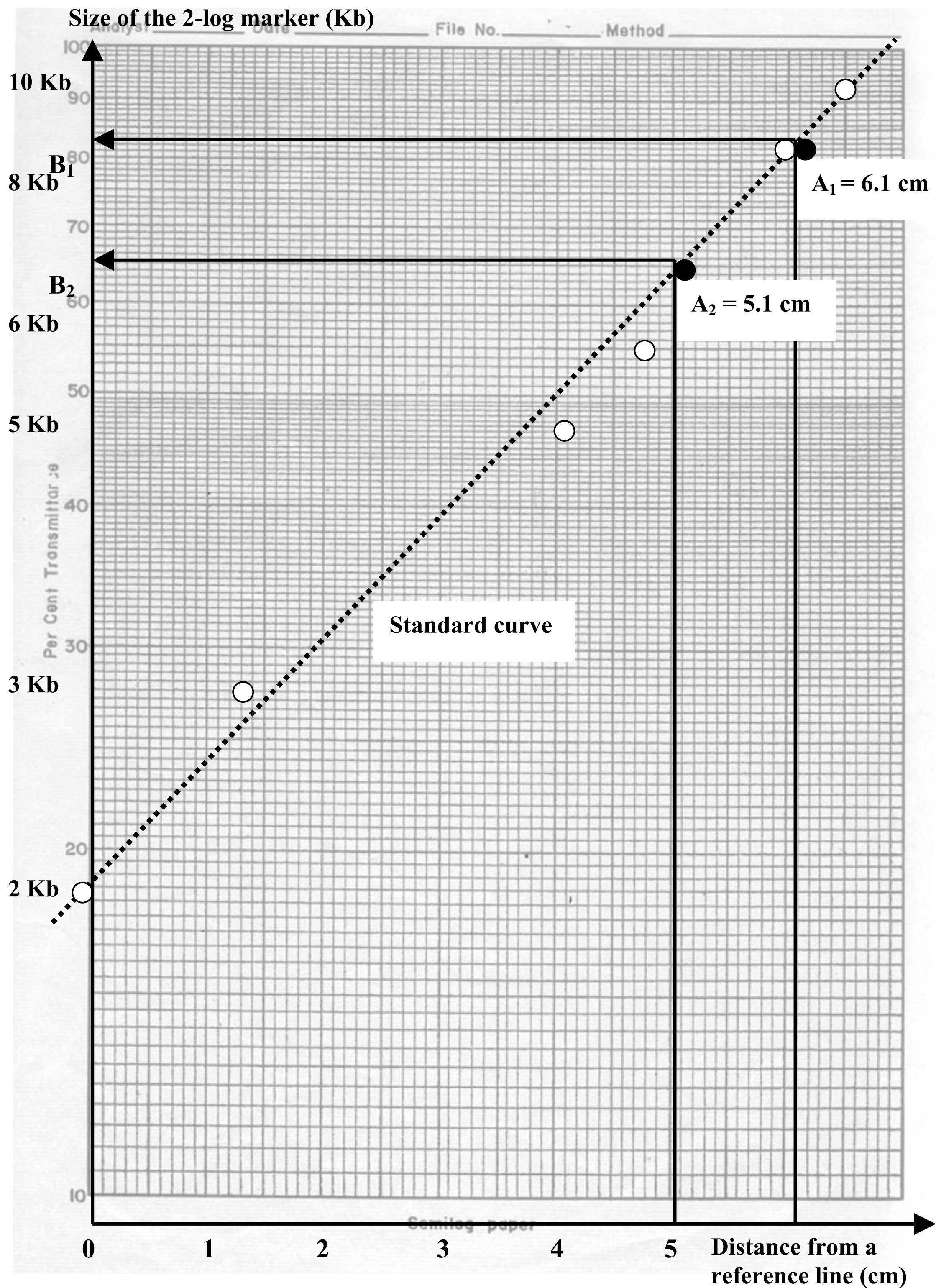


**Fig. 10 A semi-log graph showing the calculation of CGG repeats.** A *dotted line* is a standard curve. The *opened circle* is a distance between each band of marker and a reference line. The *closed circle* is a distance between bands of DNA and a reference line.

### 3.3 MS-PCR for males

Because of the various restraints on the use of Southern blot analysis (time and cost, a large amount of DNA required), MS-PCR has been developed as an alternative method for methylation analysis. Following the MS-PCR protocol developed by Weinhäusel and Haas (2001), this study was undertaken using peripheral blood DNA from 18 full mutation males, 2 premutation males and 15 normal males who had been previously diagnosed by PCR for CGG repeats and/ or Southern blot analysis. In addition, DNA from amniotic fluid culture of a full mutation male was retrospectively tested. We optimized the best PCR conditions with correctly identified methylation pattern in all samples. The expected MS-PCR product patterns are shown in Table 2.

**Table 2 Expected PCR product patterns from MS-PCR.**

| PCR product | Length (bp) | Normal male | PM male | FM male | Mosaic PM/FM male | Deletion male | Female |
|---|---|---|---|---|---|---|---|
| ***XIST* gene** | | | | | | | |
| MX | 241 | + | + | + | + | + | + |
| UX | 198 | - | - | - | - | - | + |
| ***FMR*1 gene** | | | | | | | |
| MP | 288 | - | - | + | + | - | + |
| UP | 318 | + | + | - | + | - | + |

PM = premutation, FM = full mutation.

MX and UX are *XIST* methylated and unmethylated PCR products, respectively.

MP and UP are *FMR*1 methylated and unmethylated PCR products, respectively.

All males have one active X chromosome, thus PCR product of methylated *XIST* promoter (MX) is presented in all males. All females have one active and one inactive X chromosome, thus all females exhibit both MX and UX. Individuals with normal and premutation alleles have the unmethylated *FMR*1, while full mutations have the methylated *FMR*1. In females, *XIST* is unmethylated on the inactive X chromosome, giving a methylated *FMR*1 and *XIST* is methylated on the active X chromosome, giving an unmethylated *FMR*1. In full mutation females *FMR*1 is also methylated when there are over 200 CGG repeats. Thus, all females always have four bands of PCR products. An example of MS-PCR results is shown in Fig. 11.

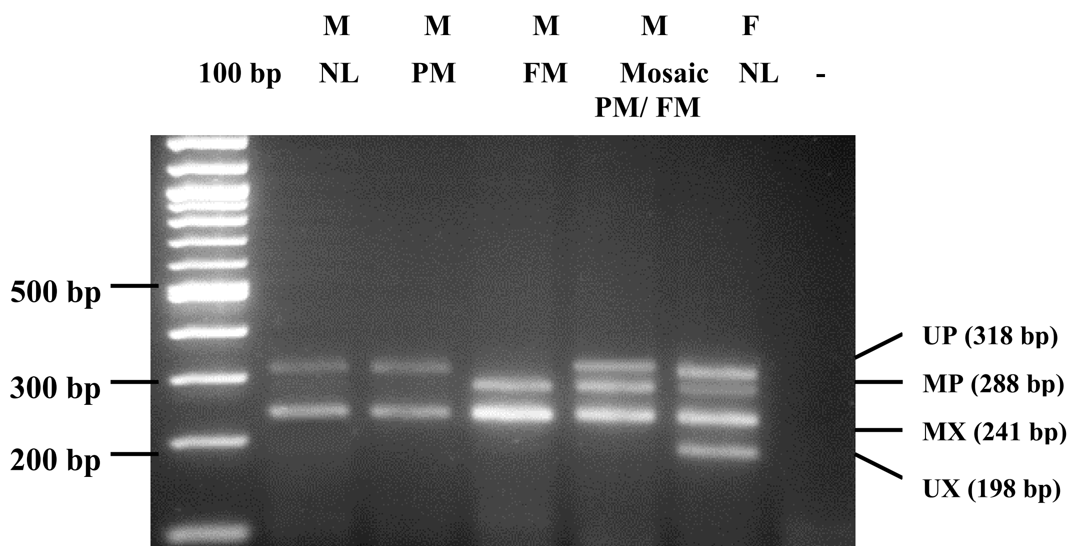


**Fig. 11 An example of results from MS-PCR for male**. From the left, lane 1 is 100 bp DNA ladder. Lane 2 is a normal male. Lane 3 is a premutation male. Lane 4 is a full mutation male. Lane 5 is a mosaic premutation/ full mutation male. Lane 6 is a normal female. Lane 7 is a negative control. UP = unmethylated *FMR*1 PCR product, MP = methylated *FMR*1 PCR product, MX = methylated *XIST* PCR product, UX = unmethylated *XIST* PCR product. M = male, F = female, NL = normal, PM = premutation and FM = full mutation.

Following the optimization of the PCR protocol, we performed a blind test using the DNA of 60 males with mental retardation or delayed development that had been referred for FXS diagnosis. Each case was simultaneously tested with standard methods. The results from MS-PCR tests revealed of 7 full mutation males and 53 normal males, which corresponded to the results from standard methods (one prenatal case was included, which showed a normal male pattern.

**3.4 MS-PCR for females**

Even though the majority of FXS cases are male, diagnosis in female should be considered. When we tested this method in females, we could not distinguish normal females from premutation or full mutation females. We modified the assay for MS-PCR of Zhoe and colleagues (2004) for diagnosis of methylation status in females. Fig. 12 to Fig.14 represent expected patterns of non-met PCR, met-PCR and mTP PCR, respectively (modified from Zhoe, et al., 2004). The optimization of PCR was undertaken on peripheral blood DNA from 5 normal females, 5 premutation females and 2 full mutation females. The results were shown in Fig.15 and Fig. 16. A prospective study was performed in one sample of amniotic fluid DNA that had referred for prenatal diagnosis. The result revealed a full mutation female, corresponding to the result from the standard methods. The number of CGG repeats greater than 350 repeats cannot be amplified, however, when combined the result of met-PCR with mTP-PCR, full mutation females can be distinguished from normal females.

The advantages of non-met PCR and met PCR showed not only the methylation status, but also the approximate number of CGG repeats. The CGG repeats could be

calculated from the size of PCR product, 168 + 3 n for non-met PCR and 109 + 3 n for met-PCR, when “n” is a size of CGG repeats.

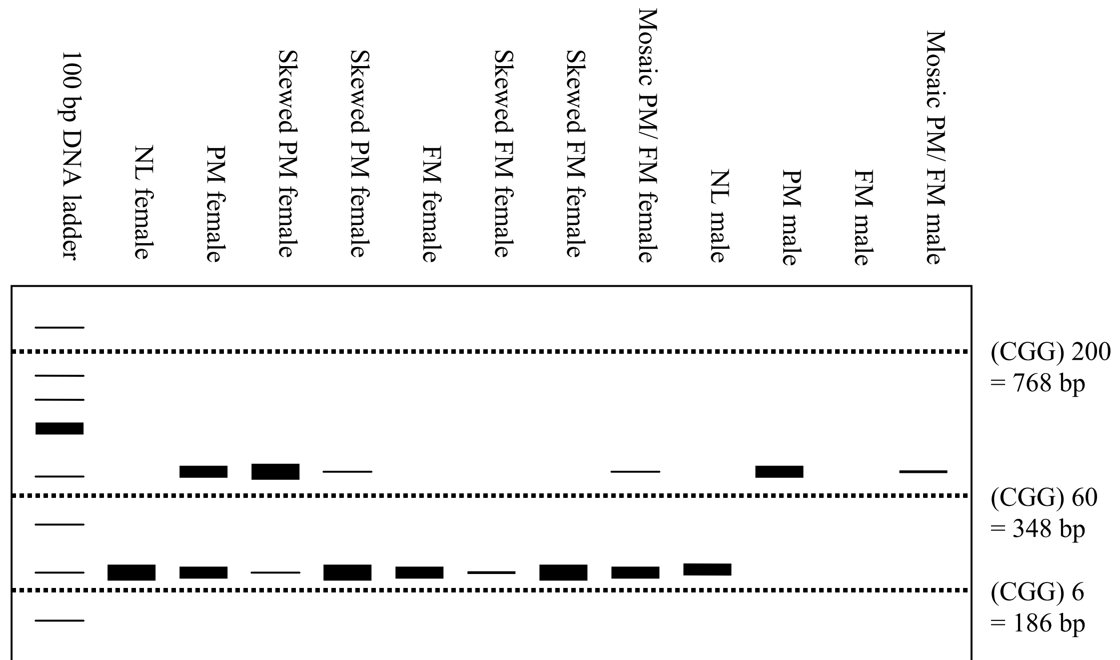


**Fig. 12 Diagrammatic presentation of the non-met PCR product patterns.** These PCR products are the result of PCR performed on an unmethylated sequence. A normal female has one or two normal bands. A premutation female has one normal band and one premutation band. A full mutation female has one normal band. Skewed X inactivation results in a difference in intensity between the two bands. A normal male and a premutation male have one normal band and one premutation band, respectively. A full mutation male has no PCR product on non-met PCR gel. M = male, F = female, NL = normal, PM = premutation and FM = full mutation.

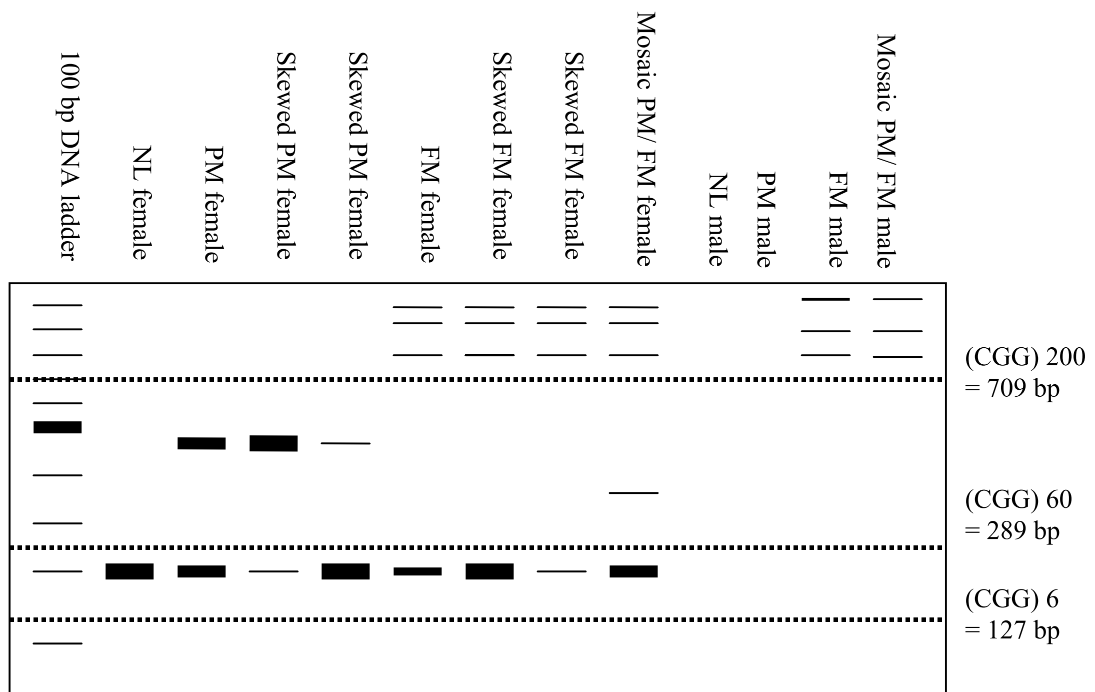


**Fig. 13 Diagrammatic presentation of the met-PCR product patterns.** These PCR products are the result of PCR performed on a methylated sequence. A normal female has one or two normal bands. A premutation female has one normal band and one premutation band. A full mutation female has one normal band and multiple bands in full mutation range. Skewed X inactivation results in a difference in intensity between the two bands. A normal male and a premutation male have no PCR product on met-PCR gel. A full mutation male has multiple bands in full mutation range. A full mutation with allele larger than 350 repeats may not be amplified by met-PCR, however it still has PCR product with mTP-PCR. M = male, F = female, NL = normal, PM = premutation and FM = full mutation.

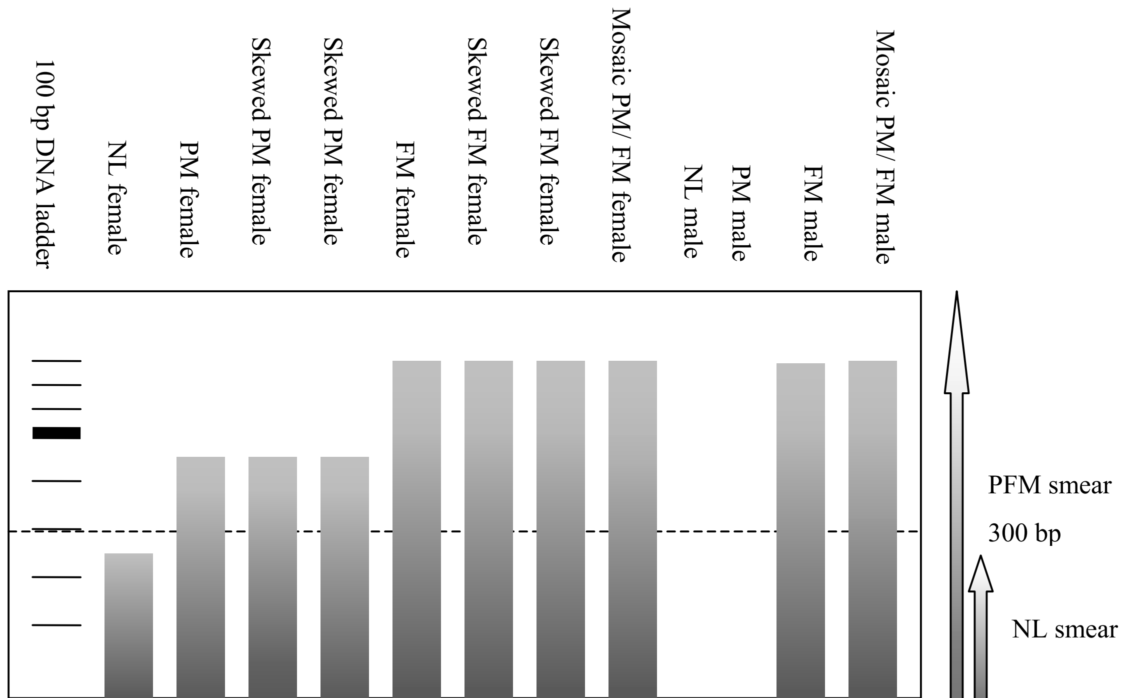


**Fig. 14 Diagrammatic presentation of mTP-PCR product patterns.** These PCR products are the result of PCR performed on a methylated sequence. A normal female has a normal methylated allele and a normal (NL) smear that not exceeds 300 bp. A premutation female and a full mutation female have a smear that exceeds 300 bp. This smear is designated as a premutation-full mutation (PFM) smear. A normal male and a premutation male have no PCR product on mTP-PCR gel because *FMR*1 allele is unmethylated. A full mutation male has a PFM smear as same as a full mutation female. Skewed X inactivation cannot be observed by this PCR. M = male, F = female, NL = normal, PM = premutation and FM = full mutation.

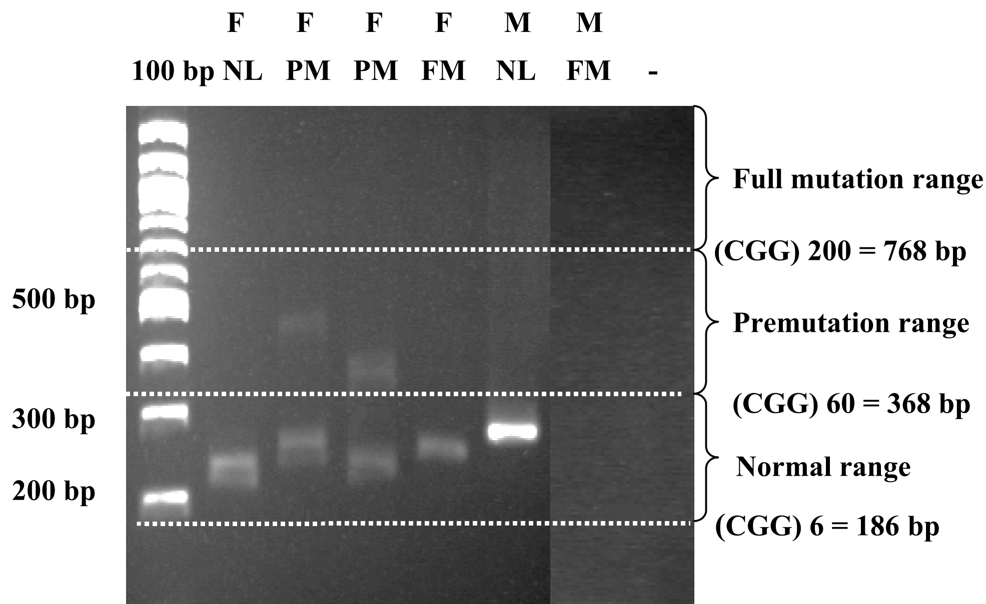


**Fig. 15 An example of results from non-met PCR.** From the left, lane 1 is 100 bp DNA ladder. Lane 2 is a normal female (20, 22 repeats), showing broad band within normal range. Lane 3 is a premutation female (29, 100 repeats), showing one band within normal range and one band within premutation range. Lane 4 is a premutation female (22, 60 repeats), showing one band within normal range and one band within premutation range. Lane 5 is a full mutation female (29, ~760, 960, 1230 repeats), showing one band within normal range but no PCR product within full mutation range. Lane 6 is a normal male (36 repeats), showing one band within normal range. Lane 7 is a full mutation male, showing no PCR product. Lane 8 is a negative control. A full mutation female in lane 5 and a normal male in lane 6 are prospective prenatal diagnosis cases. M = male, F = female, NL = normal, PM = premutation and FM = full mutation.

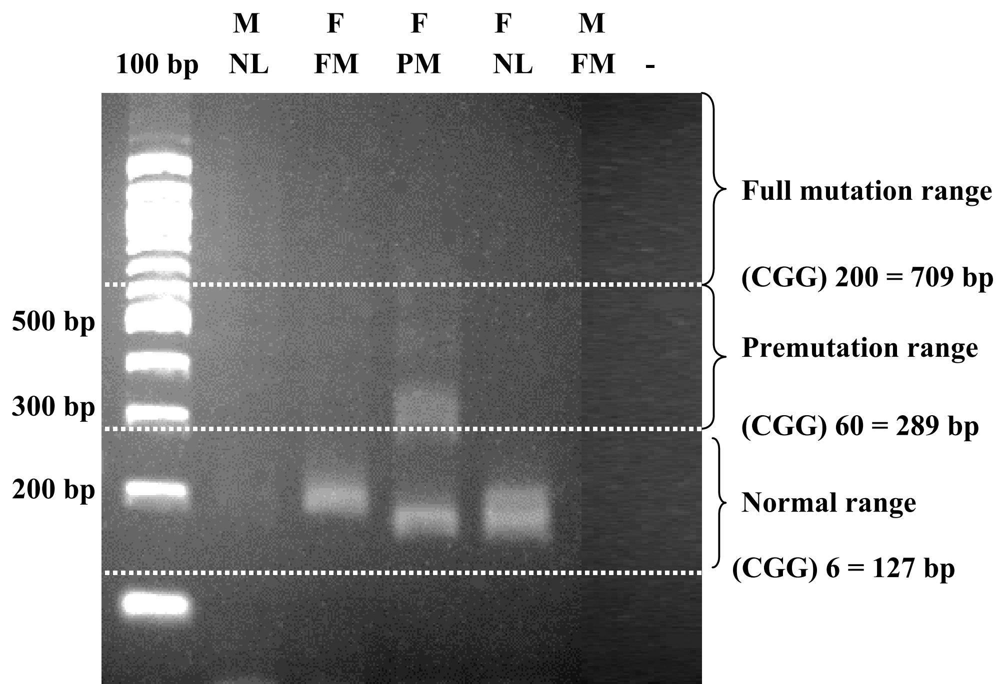


**Fig. 16 An example results from met PCR**. From the left, lane 1 is 100 bp DNA ladder. Lane 2 is a normal male (36 repeats), showing no PCR product. Lane 3 is a full mutation female (29, ~760, 960, 1230 repeats), showing one band within normal range but no PCR product within full mutation range. Lane 4 is a premutation female (22, 60 repeats), showing one band within normal range and one band within premutation ranges. Lane 5 is a normal female (20, 22 repeats), showing broad band within normal range. Lane 6 is a negative control. A normal male in lane 2 and a full mutation female in lane 3 are prospective prenatal diagnosis cases. Although there is no band within full mutation range in the full mutation female, mTP-PCR still exhibits PFM smear (Fig. 17, lane 4). M = male, F = female, NL = normal, PM = premutation and FM = full mutation.

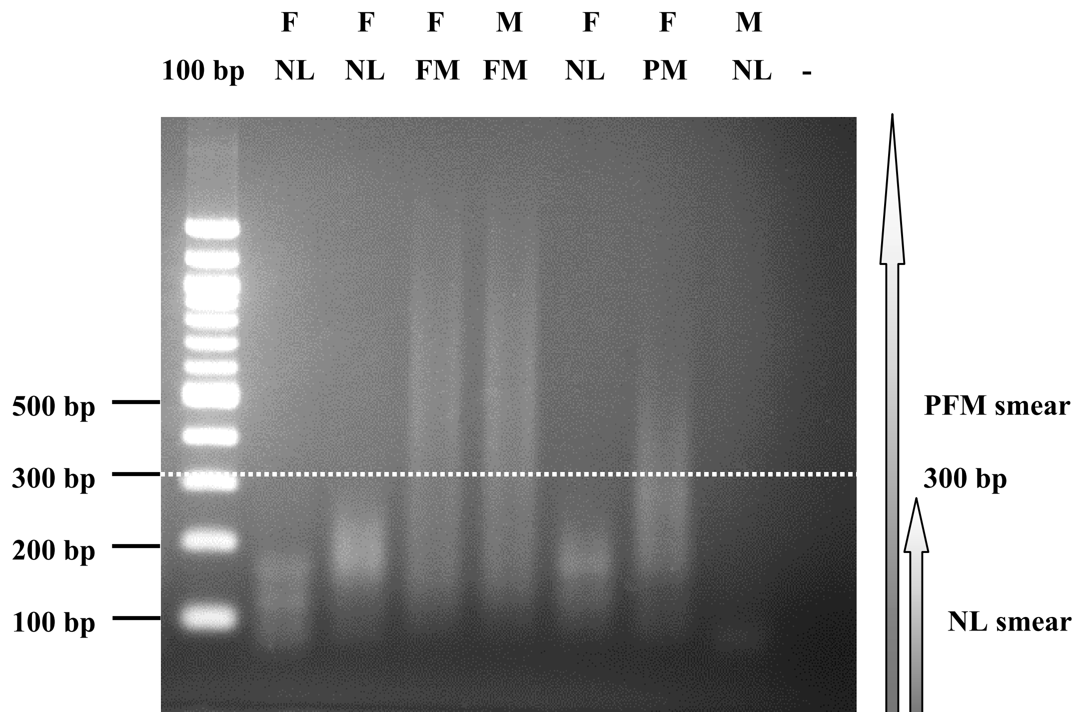


**Fig. 17 An example of results from mTP-PCR.** PCR products revealed as smear, an NL smear is a smear that not exceeds 300 bp and a PFM smear is a smear that exceeds 300 bp. From the left, lane 1 is 100 bp DNA ladder. Lane 2 is a normal female (20, 22 repeats), showing an NL smear. Lane 3 is a normal female (29, 41 repeats), showing an NL smear. Lane 4 is a full mutation female (29, ~760, 960, 1230 repeats), showing a PFM smear. Lane 5 is a full mutation male (~230, 360, 430 repeats), showing a PFM smear. Lane 6 is a normal females (22, 39 repeats), showing an NL smear. Lane 7 is a premutation female (22, 60 repeats), showing a PFM smear. Lane 8 is a normal male (36 repeats), showing no PCR product. Lane 9 is a negative control. A full mutation female in lane 4 and a normal male in lane 8 are prospective prenatal diagnosis cases. M = male, F = female, NL = normal, PM = premutation and FM = full mutation.

## Discussion

Methylation-specific PCR (MS-PCR) is a new strategy for DNA methylation analysis, without using methylation-sensitive restriction enzymes. The assay involves two steps: (1) chemical modification of DNA with sodium bisulphite which converts the unmethylated cytosine to uracil, while the methylated cytosine is not converted by this modification, and (2) PCR amplification with primers specific for the methylated versus the unmethylated DNA.

Many critical parameters affecting the specificity of MS-PCR must be considered, including complete deamination of DNA, primer design and optimal PCR conditions. The first critical step in the deamination of DNA is a complete DNA denaturation before bisulphite treatment. DNA deamination serves two purposes: (1) distinguishing between homologous DNA sequences that are different in methylation status, and (2) reducing the difficulty of amplifying across the repeats due to a high CG content. This assay converts the unmethylated cytosine to uracil that is substituted by thymidine on the PCR amplification. Besides the accurate concentration and pH of sodium bisulphite, the temperature is should be constant in every step of modification. If the temperature runs down from 55°C or incubation time less than 3 hours, it is no PCR product. The optimum time for incubation is between 4 and 6 hours.

Primer design is of great importance for a success of PCR amplification. The specificity of primers after modification should be considered. In addition, primers should incorporate enough cytosines in the original sequence to assure that unmodified DNA will not serve as a template for the primers (Herman, et al., 1998).

Weinhäusel and Haas (2001) developed a multiplex PCR assay consisting of a duplex PCR of the unmethylated and methylated sequences of the *FMR*1 and *XIST*

gene promoters as well as a duplex PCR across the unmethylated and methylated CGG repeats region. The PCR for the promoters does not apply to females because of X-inactivation. This method is unable to distinguish among normal females, premutation females and full mutation females, which reveal the same pattern. Theoretically, genotypes of females can be categorized by using the ratio analysis of the intensity of *FMR*1 promoter methylation status (UP: MP). The *XIST* promoter is methylated on the active X chromosome, resulting in unmethylation of the *FMR*1 promoter, while on the inactive X chromosome; the *XIST* promoter is unmethylated, resulting in methylation of the *FMR*1 promoter. In full mutation females, the *FMR*1 promoter can be methylated under two conditions, when it is located on the inactive X chromosomes and when there are greater than 200 CGG repeats. However, when full mutation females have non-random or skewed X inactivation toward the unmethylated *FMR*1 or methylated *FMR*1, this may affects the intensity of the PCR products, thus the ratio is changed from the ratio of full mutation females with random X-inactivation (Fig. 18-20).

The efficiency of the DNA modification with sodium bisulphite was nearly 100% under our conditions, giving a very small chance of the mis-amplification is small. Normally males have a methylated *XIST* promoter that is not converted by sodium bisulphite. The presence of an unmethylated *XIST* promoter product (UX) in male DNA sample, suggest contamination by female DNA (Fig. 21).

According to Weinhäusel and Haas (2001), MS-PCR does not predict the number of CGG repeats because the PCR amplification occurs in the promoter region which is located proximal to the CGG repeats region. Knowledge of the number of CGG

repeats is essential for genetic counseling in premutation females, because this information allows a physician to discuss the risk of having an affected offspring.

**Normal female with random X-inactivation**

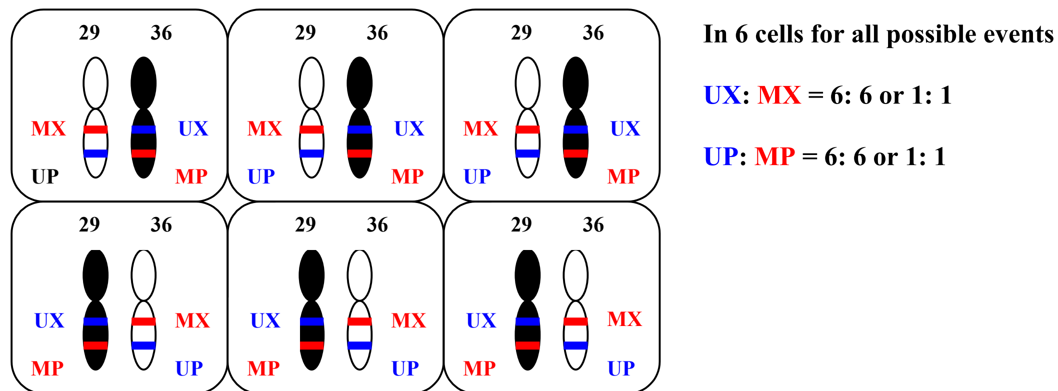


**Fig. 18 Diagrammatic presentation showing the ratio analysis of *FMR*1 unmethylated against methylated PCR products of a normal female.** The active and inactive X chromosomes are shown as white and black, respectively. The methylated and unmethylated alleles are shown as red and blue bars on the chromosome, respectively. UX and MX are PCR products of the unmeththylated and methylated *XIST* promoters, respectively. UP and MP are PCR products of the unmeththylated and methylated *FMR*1 promoters, respectively. In a normal female with two alleles of 29 and 36 CGG repeats, two events may occur with an equal proportion (random X inactivation). Two group of cells, consisting of a group of cells with 29 CGG repeats on the active X chromosome and 36 CGG repeats on the inactive X chromosome and a group of cells with 29 CGG repeats on the inactive X chromosome and 36 CGG repeats on the active X chromosome. In this case, the ratio analysis of the *FMR*1 promoter (UP: MP) is 1:1.

**FM female with random X-inactivation**

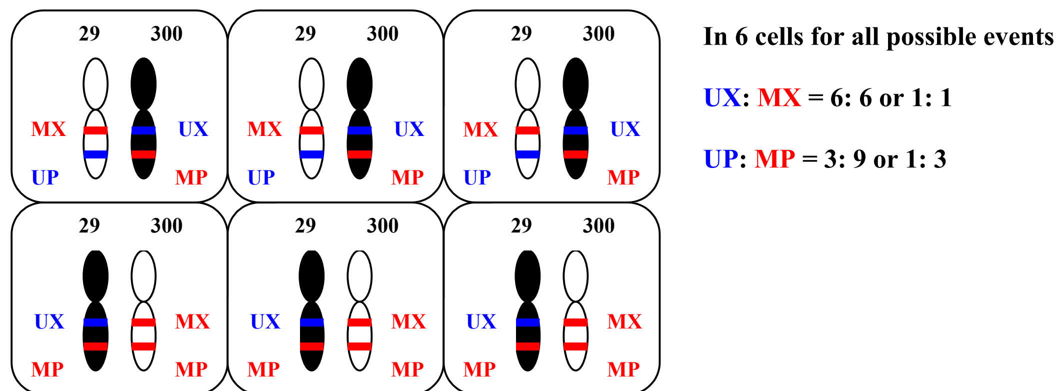


**Fig. 19 Diagrammatic presentation showing the ratio analysis of *FMR*1 unmethylated against methylated PCR products of a full mutation female.** The active and inactive X chromosomes are shown as white and black, respectively. The methylated and unmethylated alleles are shown as red and blue bars, respectively. UX and MX are PCR products of the unmeththylated and methylated *XIST* promoters, respectively. UP and MP are PCR products of the unmeththylated and methylated *FMR*1 promoters, respectively. In a full mutation female, the *FMR*1 is methylated either on the inactive normal X chromosome (29 repeats) or when there are greater than 200 CGG repeats, thus the ratio of UP: MP is 1:3. The methylation ratio of the *XIST* promoters (UX: MX) is 1:1 in all instances.

MS-PCR amplification across the repeat region allows an approximate repeat size as well as methylation status of the repeats (Zhoe, et al., 2004). Although, Weinhä usel and Haas (2001) proposed MS-PCR for the CGG repeats, but this is difficult to interpret the results in females with large premutation alleles and full mutations. Because the expanded alleles were not amplified, therefore, only the product of normal allele was observed, that is similar to product pattern of normal homozygous females (Fig. 22).

**FM female with skewed X-inactivation**

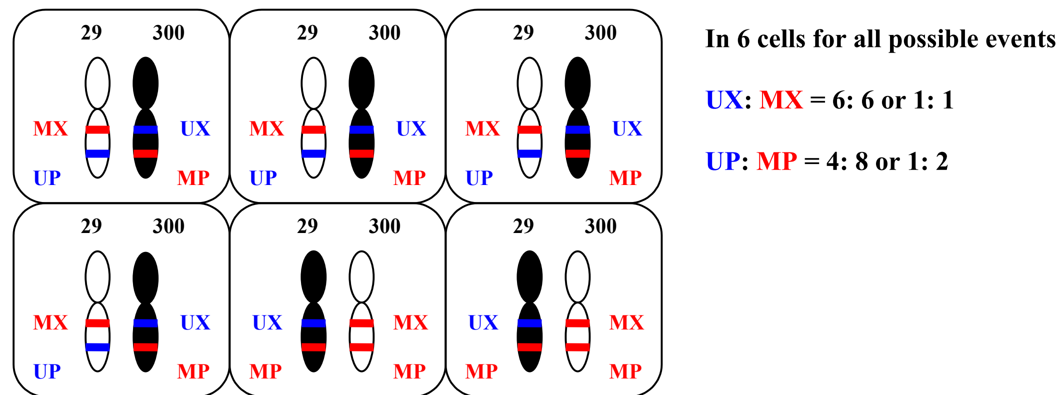


**Fig. 20 Diagrammatic presentation of the effect skewed X-inactivation on the ratio analysis of *FMR*1 unmethylated against methylated PCR products.** The active and inactive X chromosomes are shown as white and black, respectively. The methylated and unmethylated alleles are shown as red and blue bar, respectively. UX and MX are PCR products of the unmeththylated and methylated *XIST* promoters, respectively. UP and MP are PCR products of the unmeththylated and methylated *FMR*1 promoters, respectively. A full muation female with skewed X inactivation toward the active X chromosome with normal allele (CGG 29 repeats) shows the ratio of UP: MP = 1: 2.

Zhoe and colleagues (2004) have developed a triple simplex MS-PCR (non-met-PCR, met-PCR and mTP-PCR) for diagnosis of methylation status and estimating the number of CGG repeats in both males and females. Repeat size and methylation status are obtained in the same gel (either non-met PCR gel or met-PCR gel). The number of CGG repeats can be obtained from the calculation of PCR product size, but it is an

approximate number. If the exact number of CGG repeats is needed, PCR for CGG repeats must be performed.

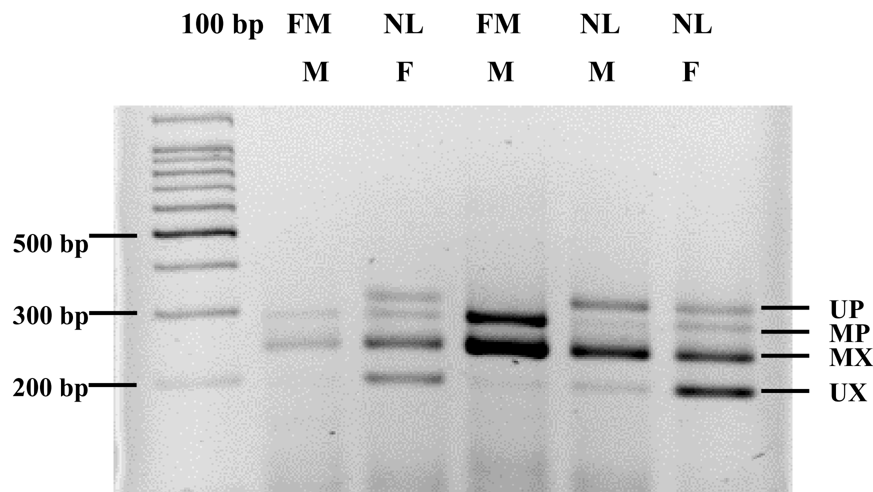


**Fig. 21 MS-PCR for males showing PCR product pattern of a normal male appearing as a female pattern.** From the left, lane 1 is a 100 bp DNA ladder. Lane 2 and 4 are full mutation males. Lane 5 is a normal male, but shows a PCR product pattern like a female (lane 3 and 6). The MS-PCR was repeated and showed a normal male pattern. The most likely cause of the first incorrect result was contamination from female DNA. UP = unmethylated *FMR*1 PCR product, MP = methylated *FMR*1 PCR product, MX = methylated *XIST* PCR product and UX = unmethylated *XIST* PCR product. NL = normal and FM = full mutation.

In our study, the triple PCR provided a possibility for screening of FXS in females. It was able to delineate normal females from premutation and full mutation females since it did not depend on the unmethylated against methylated ratio analysis. Moreover, the genotype interpretation was not affected by skewed X-inactivation. However, there were still some difficulties, for instance premutation and full mutation alleles were not amplified in the met-PCR when the CGG repeats were greater than

100 repeats. Thus we could not distinguish high CGG-premutation females and full mutation females from normal homozygous females or normal heterozygous females with small gap-size CGG repeats (i.e. 29, 30 repeats). The mTP-PCR could solve these problems. However, using only mTP-PCR, we could not distinguish low CGG-premutation females (i.e. 60 repeats) from high CGG-normal females (i.e. 50 repeats). The tests from normal females revealed a long NL smear that was similar to a short PFM smear. Moreover, mosaic premutation/ full mutation females were misdiagnosis as premutation females because only the premutation allele could be detected in non-met and met-PCR. In this case, mTP-PCR did not allow a definite diagnosis because it gave the same PFM smear. In addition, we found that the sensitivity of a non-met PCR and a met-PCR was decreased when the number of CGG repeats was increased. When we compared a met-PCR with a non-met PCR, a met-PCR was more difficult to amplify than a non-met PCR (Fig.23). An obvious premutation band is observed until the CGG of 100 repeats on the non-met PCR gel (Fig.15).

MS-PCR for diagnosis in female according to Weinhäusel and Haas (2001) is compared with Zhoe and colleagues (2004) in Table 3. Since MS-PCR does not require restriction enzymes, the MS-PCR assay can be performed on various DNA samples unsuitable for restriction digestion, such as paraffin-embedded samples, blood samples collected in a sodium heparin-treated tube, and dried blood specimens preserved on Guthrie paper (Kubota, et al., 1997; Herman, 1998). Moreover, this assay has a higher sensitivity than the Southern blot analysis which makes it more useful for methylation detection in small DNA samples. We compare MS-PCR with standard methods in Table 4.

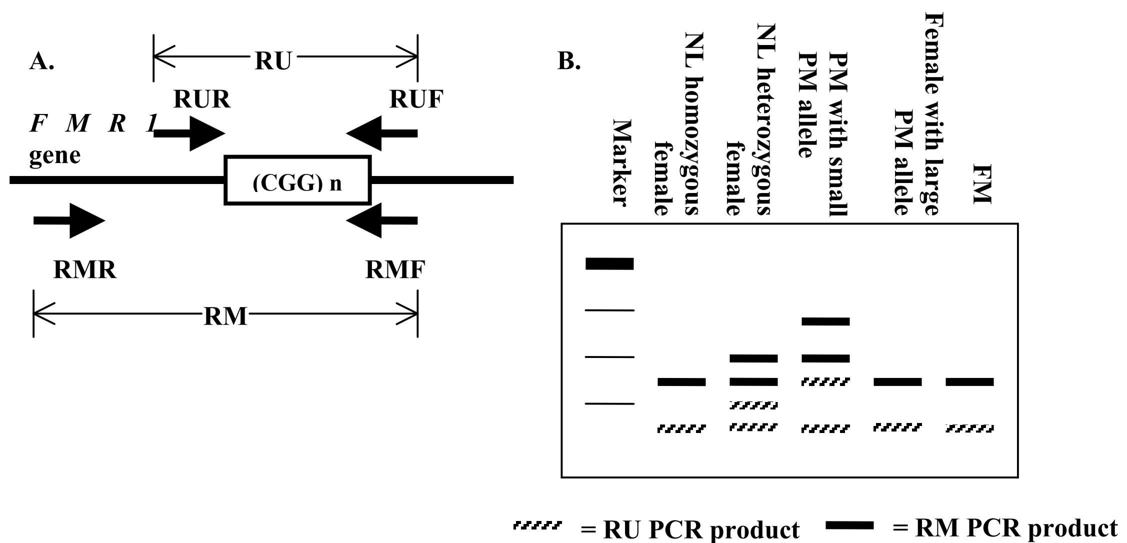


**Fig. 22 Diagrammatic presentation of MS-PCR for CGG repeats according to the assay of Weinhäusel and Haas (2001).** Amplification site of primers across CGG repeats of the *FMR*1 gene (A). RUF/ RUR and RMF/ RMR are pairs of primers for unmethylated and methylated CGG repeats, respectively. RU and RM are PCR products of unmethylated and methylated CGG repeats, respectively. In (B), patterns of PCR products are observed from gel electrophoresis. From the left, lane 1 is a marker. Lane 2 is a normal homozygous female. Lane 3 is a normal heterozygous female. Lane 4 is a premutation female. A female with a large premutation allele (lane 5) and a full mutation female (lane 6) reveal pattern of PCR product the same as in a normal homozygous female. NL = normal, PM = premutation and FM = full mutation.

There has been no previous report of prenatal diagnosis using MS-PCR. In this study, we also prospectively performed MS-PCR for prenatal diagnosis of 2 fetuses with a family history of FXS. The results showed a normal male fetus and a full mutation female fetus that corresponded to the results from the gold standard methods. Therefore, it may be possible for MS-PCR to replace Southern blot analysis for prenatal as well as postnatal diagnosis in a known FXS family. However, further study

of MS-PCR in a prenatal diagnosis should be done for validation the accuracy of this method. At the present, the prenatal and postnatal diagnosis, especially in a new case without family history of FXS, Southern blot analysis remains a gold standard for confirmation of a full mutation.

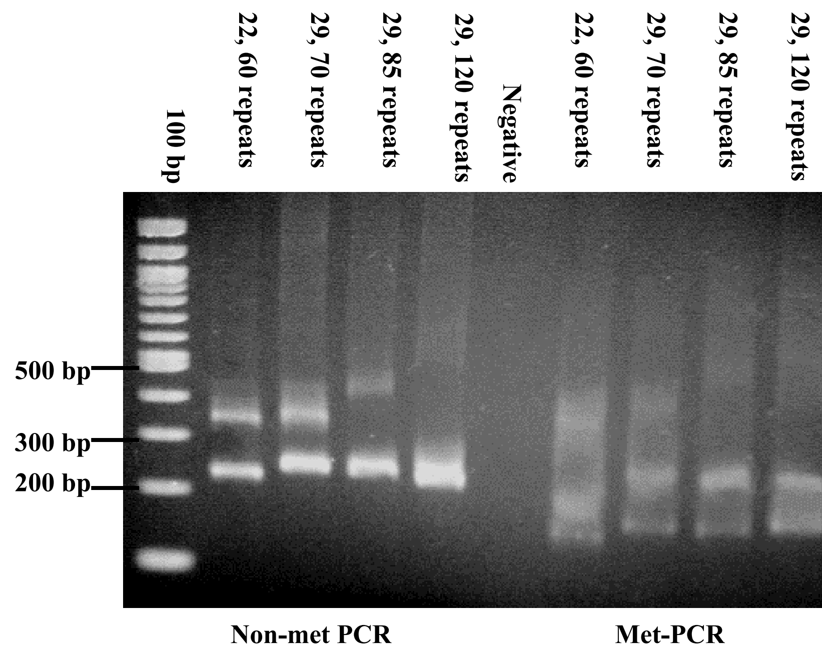


**Fig. 23 A non-met PCR compares with a met-PCR in the amplification of premutation alleles.** The results of non-met PCR are shown in lane 2-5. Lane 1 is a 100 bp DNA ladder. Lane 2 is a premutation female with 22 and 60 repeats. Lane 3 is a premutation female with 29 and 70 repeats. Lane 4 is a premutation female with 29 and 85 repeats. Lane 5 is a premutation female with 29 and ~120 repeats. Lane 6 is a negative control. The results of met-PCR are shown in lane 7-10. Lane 7 is a premutation female with 22 and 60 repeats. Lane 8 is a premutation female with 29 and 70 repeats. Lane 9 is a premutation female with 29 and 85 repeats. Lane 10 is a premutation female with 29 and ~120 repeats. Premutation alleles are easier to amplify using a non-met PCR than a met-PCR. An obvious premutation band is observed until the CGG of 85 repeats on non-met PCR gel, but of smear band on the met-PCR gel.

**Table 3 Comparison of MS-PCR for diagnosis in females according to Weinhä usel and Haas (2001) and Zhoe, et al. (2004).**

| Author | Normal female | | Premutation female | | Full mutation female | |
|---|---|---|---|---|---|---|
| | Methylation status | Repeat size | Methylation status | Repeat size | Methylation status | Repeat size |
| Weinhäusel, et al. | + | + | +/-* | +/-* | - | - |
| Zhoe, et al. | + | + | +** | +/-* | +** | - |

* Can not be diagnosed in case of large premutation allele.

** Can be diagnosed either by met-PCR or mTP-PCR.

**Table 4 Comparison of MS-PCR with standard methods for FXS diagnosis**

| Comparison items | MS-PCR | PCR for CGG repeats | Southern blot |
|---|---|---|---|
| Amount of DNA (μg) | 0.5-2 | 0.1-0.5 | 8-10 |
| Time required (days) | 2 | 2 | 5 |
| Cost | Inexpensive | Inexpensive | Expensive |
| Repeat size | Yes | Yes (normal, PM*)<br>No (FM) | Yes (FM*)<br>No(normal, PM) |
| Methylation status | Yes | No | Yes |

PM = premutation, FM = full mutation

* Approximate repeat size