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## Establishment of a Nonradioactive Molecular Diagnosis of Fragile-X Syndrome\*

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**Abstract:** Fragile-X syndrome is a hereditary dynamic mutation disorder, predominantly caused by a large expansion of CGG trinucleotide repeats in the FMR 1 gene leading to methylation and down regulation of transcription of the gene. For the molecular diagnosis of the disease, the repeat locus in FMR 1 gene is primarily detected by Southern blotting with radioactively labeled probes. Unusually GC rich composition of the expanded region caused technical difficulties during PCR based testing and therefore is not performed by most diagnostic laboratories. We established a Southern blot method and a novel PCR protocol which enables the amplification of normal, premutated and full

mutated alleles. In both techniques bands are visualized by digoxigenin labeled probe and chemiluminescent detection. Although diagnosis of fragile-X was possible by cytogenetic analysis in our division, it was not possible to diagnose premutation carriers or mosaic fragile-X patients. This newly established nonradioactive PCR and Southern blot analysis will provide routine detection of full fragile-X mutations and premutations in our laboratory and therefore enable us to offer accurate genetic counseling and prenatal diagnosis.

**Key Words:** Fragile-X, molecular diagnosis, nonradioactive labeling, polymerase chain reaction.

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### Introduction

Fragile-X syndrome is an X-linked dominant disorder with reduced penetrance in females and characterized by moderate to severe mental retardation, large head, long face, large ears, and large testicles (macroorchidism). It is recognized as the most prevalent form of inherited mental deficiency. The overall prevalence is estimated to be about 1:4000 for males and 1:6000 for females (1).

It has been elucidated that the silencing of the FMR 1 gene, located at Xq 27.3, is responsible for the disease (2, 3). The FMR 1 gene was cloned and characterized in 1991 (4-8).

The gene contains unstable repeat sequences of (CGG)<sub>n</sub> at 5'-untranslated region in exon 1. In a normal population CGG repeat is polymorphic and varies in length from 6-50 repeats (9). In phenotypically normal carriers the expanded repeat numbers range from 50-200 and become unstable (premutation). When unstable, the copy number changes during transmission from parent to

child. This process is termed dynamic mutation. In most fragile-X patients the CGG repeat is significantly increased in length to more than 200 repeats (full mutation) (5,7,9,10). The change from normal copy number to full mutation is a multi step process proceeding through premutation steps, rather than a single event characteristic of classical mutation. In addition, an upstream promoter region of the gene, CpG island, is abnormally methylated in most affected individuals (4,6). This finding led to the hypothesis that expansion to full mutation size causes the hypermethylation of this promoter region and thereby inactivates the FMR1 gene. Amplification of CGG repeats and abnormal methylation show a correlation with affected status.

Diagnosis of fragile-X syndrome has been available by means of cytogenetic analysis for many years. However, there have been some problems in identifying mosaic males, premutations and some full mutation females (11). For these reasons, alternative methods have been pursued in many molecular laboratories during the past

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decade. The recently developed FMR protein antibody test allows rapid detection in male patients using blood smears and is found to be a suitable screening test for the population of retarded males (12-13). Presently two molecular methods are preferred for the detection of patients and carriers of fragile-X syndrome (14-16). The most widely used protocol is direct Southern blot hybridization of genomic DNA digested with a pair of restriction enzymes, one of which is methylation sensitive (17). In this method, the extent of amplification and level of methylation can be simultaneously detected. The other protocol is the use of polymerase chain reaction (PCR) to rapidly detect the size of the repeat expansion (18,19). Compared with the well-established Southern blot method, the PCR protocol contains major technical difficulties in amplifying higher premutated and full mutated alleles due to strong secondary structures of this region.

We describe here the establishment of Southern blot and PCR methods for the molecular analysis of fragile-X syndrome in our laboratory. In both methods nonradioactive DIG system was used for the detection (20, 21). A novel PCR method based on the use of Expand Long PCR system was designed to amplify normal, premutated and full mutated alleles. This is the first report in Turkey of nonradioactive molecular diagnosis of fragile-X syndrome and detection of full mutation males and females by a PCR based method.

## Materials and Methods

### Fragile-X Families

We analyzed 32 DNA samples isolated from the blood samples of eight fragile-X affected families that had been clinically and cytogenetically assessed in our clinic (22). For the control of our molecular results, part of the DNA samples were sent to the Center for Human Genetics in Leuven, Belgium, for DNA analysis by Southern blot (17).

### DNA Isolations

5 ml of peripheral blood collected in K<sub>3</sub>EDTA tubes was used from each subject. DNA was isolated with a DNA isolation kit (Boehringer Mannheim).

### Genomic Southern Hybridization

Genomic DNA (5µg) was digested with both EcoR1 (50 U, Boehringer Mannheim) and methylation sensitive enzyme EclX1 (isochimer of Eag1, 25U, Boehringer Mannheim). Digestion products were separated on an agarose gel (0.7 %) along with DIG-labeled molecular weight marker (MW VII, Boehringer Mannheim) and

transferred to a nylon membrane (positively charged) following denaturation and neutralization procedures. DNA was crosslinked to the membrane by UV light. Blot was prehybridized for 4 hours, then hybridized with DIG labeled pFxa1NHE probe (Oncor) in DIG Easy Hyb buffer (Boehringer Mannheim) at 60 °C shaking in a hybridization oven (Hybaid) for 16 hours. After stringent washing of the membrane, the bands were detected according to the procedures described in the DIG Luminescent Detection kit (Boehringer Mannheim). Incubation of CSPD was performed at 37 °C for 15 min, and the membrane was exposed to X-ray film for 16-18 hours.

### PCR Assay

Amplification of the CGG repeat region of normal, premutated and full mutated alleles in the FMR1 gene was carried out by PCR reaction with a total volume of 25 µl, containing 200 ng of genomic DNA, 300 nm of each primer (23), 5 % of DMSO, 5 % of glycerol, Expand Long PCR buffer 1 (Boehringer Mannheim), 350 µM of each of dATP, dCTP, and dTTP, 350 µM of 7-deaza dGTP and 1 U Expand Long Template PCR Kit Enzyme Mix (Boehringer Mannheim). Denaturation was performed at 96 °C for 2 min. The samples were then subject to 10 cycles of amplification (96 °C 30 s; 54 °C 30 s; 68 °C for 8 min) in a thermocycler (MJ Research). The reaction was stopped on ice, following the last denaturation for further addition of an enzyme (1U), and continued for 30 more cycles (96 °C 30 s; 52 °C 30 s; 68 °C for 8 min). The reaction was finalized by keeping the reaction at 68 °C for 12 min then 4 °C. The PCR products were run on 1.2 % agarose (molecular screening, Boehringer Mannheim) gel. Blotting procedure, hybridization and chemiluminescent detection were performed as mentioned above for genomic southern hybridization. The only difference was that the hybridization buffer contained digoxigenin-labeled 5'-(CGG)5-3' probe (5 pmol/ml) instead of the above mentioned Oncor probe. X-ray exposure was approximately 1-2 hours.

For the indirect screening test, which enables the amplification of normal alleles only, a similar PCR protocol was used except 100 % 7-deaza dGTP was replaced by 75 % 7-deaza dGTP, the elongation time was reduced to 3 minutes, the reaction was not interrupted for extra addition of an enzyme and bands were visualized by ethidium bromide staining.

## Results

Overall, we tested 32 subjects of which 13 were analyzed by both techniques. The total number of

samples tested by the Southern blot method was 21 and by the PCR method 24. Tested subjects versus status of the individuals are outlined in Table 1. The results obtained from Southern blot and PCR were in agreement with the results provided by the Center for Human Genetics in Leuven, Belgium.

Although our primary aim was to establish a convenient PCR method for the molecular analysis of fragile-X families, we also wanted to set up a Southern blot method for comparison of our PCR results since the latter is a common method used by most diagnostic laboratories and possesses an advantage of providing information regarding the methylation status of the promoter region.

### Genomic Southern Hybridization

The strategy used for Southern blot analysis is illustrated in Figure 1. *EcoR1* and methylation sensitive enzyme *EciX1* (isochimer of *Eag1*) digest of genomic DNA gives the band pattern shown in Table 2. *EcoR1/EciX1* double digest gave a normal band size of 2.8 kb for normal males. In normal transmitting males the size of these bands was increased to 3.4 kb. In affected males, band size was increased above 5.8 kb due to the addition of more than 200 repeats causing methylation of the CpG islands. In premutation and full mutation carrier females the band pattern was similar to males but there were additional bands contributed by a second X chromosome. In normal females two fragments were detected: 5.2 kb (inactive X chromosome) and 2.8 kb (active X chromosome) (17, 24). In mutational mosaic males or females, the expected band sizes are variable reflecting some cells with full mutation which are fully methylated and some cells with premutation with no methylation (15).

Typical results of normal and fragile-X subjects from Southern blot analysis by DIG labeled pFxa1NHE probe

are illustrated in Figure 2. The premutation carrier mother (lane 3) had two band patterns as expected between 2.8-3.4 kb and 5.2-5.8 kb regions. Her daughter (lane 1) was found to have full mutation showing one normal allele at 2.8 kb and a full mutated allele, not clearly visible in this photograph, at 7.5 kb, indicated by the arrow. Her son (lane 2), had a full mutated allele at 7.5 kb range. Normal male and normal female band patterns were as expected (lanes 4 and 5 respectively). Normal transmitting male (lane 6) had band sizes of 2.8 kb and 4.0 kb. The analysis result of this patient complies with the mutational mosaic individuals stated to contain some cells with the normal number of repeat copies while some other cells with premutation or full mutation copy numbers (15).

### PCR

The amplification of the CGG locus of the FMR1 gene is not possible by classical PCR conditions. Therefore, the conditions that affect the amplification of CG rich regions (denaturation temperatures, elongation times, DMSO and glycerol concentrations, addition of 7-deaza dGTP) had to be modified. In addition, annealing temperatures, enzyme concentration, and primers also play an important role for specific amplification of this region. In our study, we tried 6 different primers in 9 different combinations under various PCR conditions (23, 25, 26). One set of forward and reverse primers were elected to provide the specific amplification (23). Normal repeats were amplified with 75 % 7- deaza dGTP and visualized by ethidium bromide staining. The typical result of this study is shown in Figure 3. Amplification bands of normal male were seen while full mutation male (lane 6) was not visualized. This method can be used as a simple test for indirect screening to eliminate normal males from further analysis. However, it may give false negative results for mosaic males, therefore should not be preferred for routine application.

Status	Tested Subjects Total 32		
	Only by Southern Blot	Only by PCR Test	Southern Blot + PCR Test
Normal (7)	2	1	4
Premutation (15)	5	6	4
Full mutation (10)	1	4	5

Table 1. The outline of total type of tests versus status of the subjects.

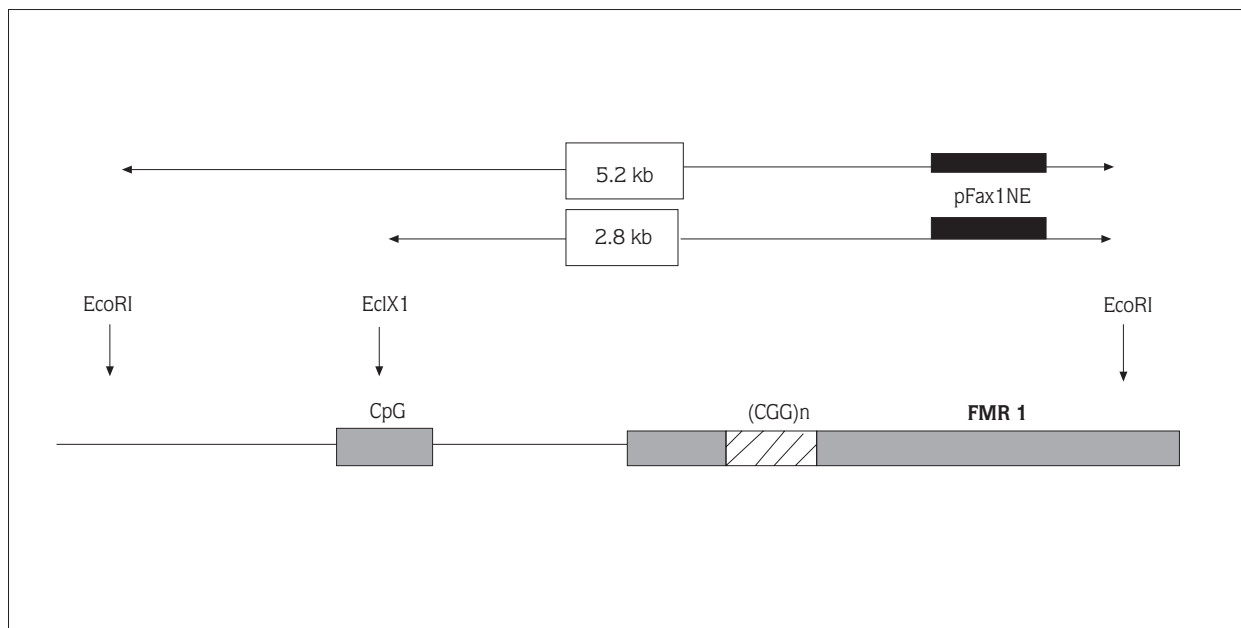


Figure 1. FMR 1 gene region with the area of size amplification, restriction sites with pFax1NE probe. EcoR1 gives a normal band size of 5.2 kb. DNA of normal male is unmethylated and gives a 2.8 kb band from EcoR1/EclX1 double digestion. In nontransmitting males the size of these bands is increased by the length of the -CGG- repeats. In affected males, *EclX1* does not cut methylated DNA then *EcoR1/EclX1* digestion gives only an identical band to that of *EcoR1* digestion alone (5.2 kb plus the size of the amplification). Mosaic males for premutation and full mutation gives the combination of unmethylated and methylated patterns depending upon fragment size. In normal females the promoter region is methylated on inactive X chromosome and therefore two fragments are detected: 5.2 kb from inactive X and 2.8 kb from active X. In premutation and full mutation females, amplified bands are like those in males, but there are additional bands contributed by the second X chromosomes.

Type of mutation (CGG repeats)	Genomic Southern Hybridization (kb)		PCR Test (bp)
	MALE	FEMALE	
Normal (6-50)	2.8	2.8 + 5.2	480-612
Premutation (50-200)	2.8- 3.4	2.8; 2.8-3.4 5.2; 5.2-5.8	613 - 1062
Full Mutation (> 200)	> 5.8	2.8 5.2; > 5.8	> 1062
Mosaic (50-200 + > 200)	2.8; 2.8-3.4 > 5.8	2.8; 2.8-3.4 5.2; > 5.8	480-612 612-1062; > 1062

Table 2. Size expected from genomic Southern hybridization and PCR test for normal, premutation and affected individuals.

Reliable amplification of premutation and full mutation alleles were successful only when a high denaturation temperature (96 °C) and 100 % 7-deaza

dGTP are used with 5 % DMSO and 5 % glycerol. The efficiency of the reaction was obtained by readdition of the enzyme after the first 10 cycles of the PCR since high

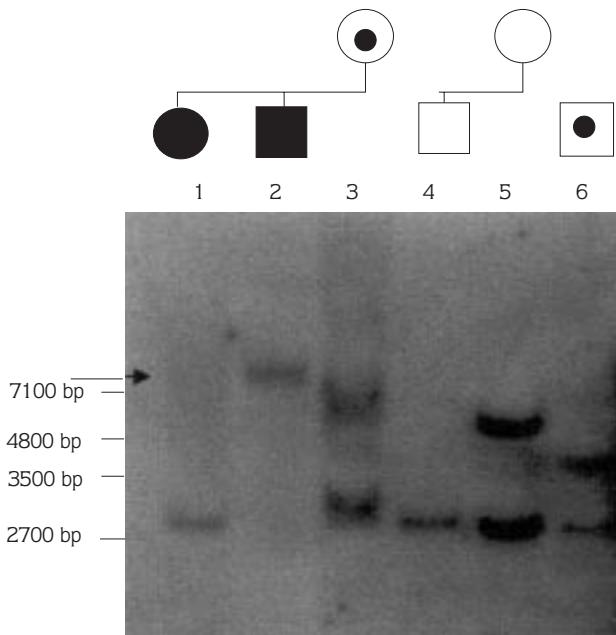


Figure 2. Southern blot analysis with DIG labeled probe pFxaNE and chemiluminescent detection of fragile-X family members. Lane 1: full mutation carrier female, lane 2: full mutation male, lane 3: premutation carrier female, lane 4 and 5: normal transmitting male and normal female, respectively. Lane 6: normal transmitting mosaic male. The sizes in bp are shown on the left. Pedigree symbols are as follows: open circle, normal female; open square, normal male; dot in square, normal transmitting male; dot in circle, female premutation carrier; striped circle, female with full mutation; shaded square, full mutated male.

denaturation temperatures partially inactivate the enzyme. PCR products were visualized by DIG detection system following DIG labeled oligonucleotide probe hybridization. The expected band pattern of PCR protocol is listed in Table 2. According to our PCR protocol, normal alleles of 6-50 repeats gave 480-612 bp, premutation of 50-200 repeats gave 613-1062 bp and full mutation of more than 200 repeats gave bands bigger than 1062 bp. Depending on the mutational variety of the cells, mosaics are expected to give bands at normal size as well as premutation and full mutation sizes. Typical results of the analysis of a fragile-X family by PCR are illustrated in Figure 4. On the X-ray film, PCR results of two fragile-X families are shown. Premutation carrier mother (lane 4), had one amplification band for normal allele of 537 bp correlated to 25 repeats and another major amplification band at 741 bp correlated with 93 repeats. Her full mutated son (lane 2) had a smear above 1500 bp which correlated with more than 345 repeats; and her full mutated daughter (lane 3) showed one normal allele size with 537 bp correlated to 25 repeats and smear above 1288 bp which correlated with repeats above 274. The sister of this mother (lane 1) was also found to be a premutation carrier with normal allele size of 562 bp correlating to 32 repeats and other major band at 944 bp exhibited expanded allele size of 159 repeats. The second family was representative for two premutation carrier females, mother and daughter, (lanes 5 and 6 respectively) with normal allele amplifications around 524 bp correlated to 21 repeats

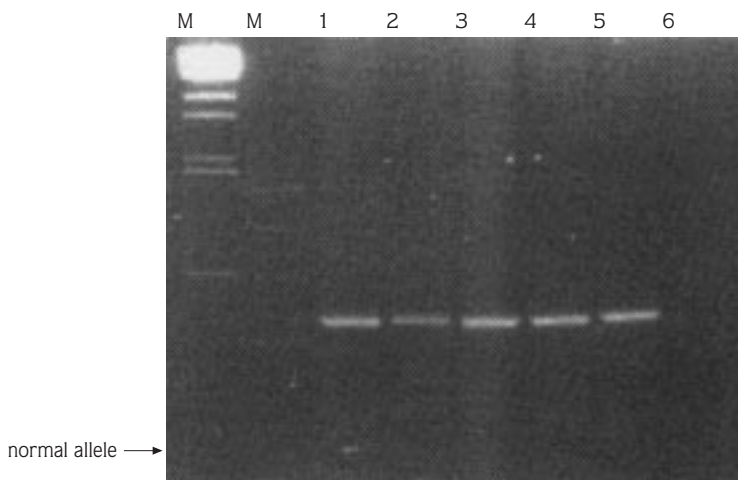


Figure 3. The results of simple PCR amplification with 75 % 7-deaza dGTP protocol of normal male (lanes 1-5) and full mutation male (lane 6) subjects. The bands are separated on 1 % agarose gel and stained with ethidium bromide. Amplified normal alleles were visualized while no amplification band was observed with full mutation males. M indicates lane the marker.

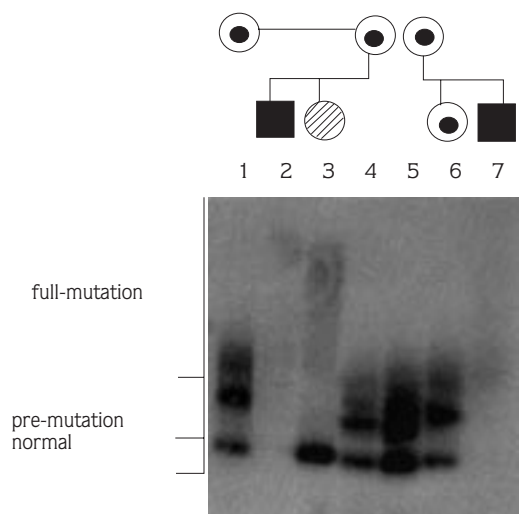


Figure 4. Expand Long PCR assay and chemiluminescent detection of normal, premutated and full mutated alleles in two unrelated fragile-X families. Lanes 1, 4, 5 and 6: pre-mutation carrier female, lanes 2 and 7: full mutation males, lanes 3: full mutation carrier female. The sizes in bp are shown on the left. The size range of normal, premutated and full mutated alleles are shown on the right. Pedigree symbols are as follows: dot in circle, female pre-mutation carrier; striped circle, female with full mutation; shaded square, full mutated male.

and expanded allele size at 754 bp with 97 repeats and 794 bp with 109 repeats, respectively. Her full mutated son (lane 7) exhibited one major band at 1318 bp corresponding to 285 repeats.

### Discussion

Diagnosis of the fragile-X syndrome is generally applied by cytogenetic and molecular techniques. Although molecular diagnosis is rapidly replacing cytogenetic diagnosis because of its lower cost and greater reliability, cytogenetic analysis may still be needed to confirm the clinical diagnosis of the index case by excluding other chromosomal abnormalities in a newly diagnosed fragile-X family.

The two molecular methods currently used for detection of fragile-X mutations are Southern blot hybridization and PCR analysis. The main advantage of

Southern blot is its ability to discriminate methylation sensitivity simultaneously with the expanded size of the region. However, this accessory has now been overtaken by new studies on PCR (27). The advantage of PCR is that it is rapid, the materials are generally less expensive and size resolution is more accurate. The simple PCR protocol with 75 % 7-deaza dGTP and ethidium bromide staining can separate normal males from fragile-X males and therefore could be used as an indirect screening test for nonspecific mental retardation cases. Since two alleles are expected from females, this test is not suitable as a screening test for females. In addition, this simple test may give false negative results for normal transmitting males and mosaic males, and therefore is not an appropriate test for further application on clinically and cytogenetically diagnosed fragile-X families.

Amplification of the CG rich regions is technically very difficult due to the secondary structures of this region. Modification of the critical parameters of the classical PCR protocol, like replacing dGTP with 7-deaza dGTP, adding DMSO and glycerol, using high denaturation temperatures and adding fresh enzyme during the reaction, help to provide better denaturation of the region while keeping the enzyme active and stable for amplification. In our established PCR method, alleles carrying full mutation appear as a smear around a condensed band, probably due to further denaturation problems of the strong secondary structures of this region. Sometimes, alleles carrying pre-mutation appear as a major band surrounded by minor bands. These bands may either reflect true heterogeneity of genomic DNA isolated from peripheral blood or be produced due to recombination artifact during PCR amplification (28).

When analyzing an identified fragile-X family, both direct genomic hybridization and PCR analysis are currently used for the diagnosis of fragile-X syndrome. Our next aim is to establish a methylation sensitive PCR method to overcome the need for Southern blot.

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## References

1. Turner G, Webb T, Wake S, Robinson H. Prevalence of fragile-X syndrome. *Am J Med Genet* 64: 196-7, 1996.
2. Richards R I, Holman K, Kozman H, Kremer E, Lynch M, Pritchard M, Yu S, Mulley J, Sutherland G R. Fragile-X syndrome: genetic localization by linkage mapping of two microsatellite repeats FRAXAC1 and FRAXAC2 which immediately flank the fragile site. *J Med Genet* 28: 818-23, 1991.
3. Siomi H, Siomi M C, Nussbaum R L, Dreyfuss G. The protein product of the fragile-X gene, FMR1, has characteristics of a RNA binding protein. *Cell* 74: 291-8, 1993.
4. Oberle I, Rousseau F, Heitz D, Kretz C, Devys D, Hanauer A, Boue J, Bertheas M F, Mandel J L. Instability of a 550 base pair DNA segment and abnormal methylation in fragile-X syndrome. *Science* 252: 1097-102, 1991.
5. Kremer E J, Yu S, Pritchard M, Nagaraja R, Heita D, Lynch M, Baker E, Hyland V J, Litle R D, Wada M, et al. Isolation of a human DNA sequences which spans the fragile-X. *Am J Hum Genet* 49: 656-61, 1991.
6. Bell M V, Hirst M C, Nakahori Y, MacKinnon R N, Roche A, Flint T J, Jacobs P A, Tommerup N, Tranebjaerg L, Froster-Iskenius U, et al. Physical mapping across the fragile-X: hypermethylation and clinical expression of the fragile-X syndrome. *Cell* 64: 861-66, 1991.
7. Heitz D, Rousseau F, Devys D, Saccone S, Abderrahim H, Le Paslier D, Cohen D, Vincent A, Toniolo D, Della Vella G, et al. Mandel J L. Isolation of sequences that span the fragile-X and identification of a fragile-X related CpG island. *Science* 251: 1236-39, 1991.
8. Eichler E E, Richards S, Gibbs R A, Nelson D L. Fine structure of the human FMR 1 gene. *Hum Mol Genet* 11: 1973-74, 1993.
9. Fu Y H, Kuhl D P A, Pizzuti A, Pieretti M, Sutcliffe J S, Richards S, Verkerk A J M H, Holden J J A, Fenwick Jr R G, Warren S T, et al. Variation of the CGG repeat at the fragile X site results in genetic instability: resolution of the Sherman paradox. *Cell* 67: 1047-58, 1991.
10. Yu S, Pritchard M, Kremer E, Lynch M, Mancarrow J, Baker E, Holman K, Mulley J C, Warren S T, Schlessinger D, et al. I. Fragile-X genotype characterized by an unstable region of DNA. *Science* 252: 1179-81, 1991.
11. Tarleton J C, Soul R A. Molecular genetic advances in fragile-X syndrome. *J Pediatrics* 3: 122-85, 1993.
12. Willemsen R, Smits A, Mohkamsing S, van Beerendonk H, de Haan A, de Vries B, van den Ouweland A, Sistermans E, Galjaard H, Oostra B A. Rapid antibody test for diagnosing fragile X syndrome: a validation of the technique. *Human Genet* 99(3): 308-11, 1997.
13. de Vries B B, Mohkamsing S, van den Ouweland A M, Halley D J, Niermeijer M F, Oostra B A, Willemsen R. Screening with the FMR 1 protein test among mentally retarded males. *Hum Genet* 103(4): 520-2, 1998.
14. Hecimovic S, Barisic I, Müller A, Petkovic I, Baric I, Ligutic I, Pavelic K. Expand Long PCR for fragile X mutation detection. *Clin Genet* 52: 147-54, 1997
15. Sutherland G R, Mulley J C. Fragile X syndrome and fragile XE mental retardation. *Prenatal Diagnosis* 16: 1199-1211, 1996.
16. Tunçbilek E, Alikışifoğlu M, Boduroğlu K, Aktaş D, Anar B. Fragil X sendromlu 12 vakanın klinik bulguları. *Çocuk Sağ Hast Der* 41: 31-38, 1998.
17. Rousseau F, Heitz D, Biancalana V, Blumenfeld S, Kretz C, Boue J, Tommerup N, Hagen C V D, de Lozier Blanchet C, Croquette M F, et al. Direct diagnosis by DNA analysis of the fragile-X syndrome of mental retardation. *New Eng J Med* 325(24): 1673-1681, 1991.
18. Pergolizzi R G, Erster S H, Goonewardena P, Brown W T. Detection of full fragile X mutation. *The Lancet* 339: 271-2, 1992.
19. Erster S H, Brown W T, Goonewardena P, Dopkin C S, Jenkins E C, Pergolizzi R G. Polymerase chain reaction analysis of fragile X mutations. *Hum Genet* 90: 55-61, 1992.
20. Nanba E, Kohno Y, Matsuda A, Yano M, Sato C, Hashimoto K, Koeda T, Yoshino K, Kimura m, Maeoka Y, et al. Non-radioactive DNA diagnosis for the fragile X syndrome in mentally retarded Japanese males. *Brain Dev* 17(5):317-21, 1995.
21. el-Aleem AA, Bohm I, Temtamy S, el-Awady M, Awadalla M, Schmidtke J, Stuhmann M. Direct molecular analysis of the fragile X syndrome in a sample of Egyptian and German patients using non-radioactive PCR and southern blot followed by chemiluminescent detection. *Hum Genet* 96(5):577-84, 1995.
22. Sutherland G R. Heritable fragile sites on human chromosomes. I. Factors affecting expression in lymphocyte culture. *Am J Hum Genet* 31: 125-35, 1979.
23. Cao J, Tarleton J, Barberio D, Davidow L S. A simple fragile X PCR assay with 7-deazaguanine-substituted DNA visualized by ethidium bromide. *Mol Cell Probes* 8: 177-80, 1994.

24. Rousseau F, Heitz D, Oberle I, Mandel JL. Selection in blood cells from female carriers of the fragile X syndrome: inverse correlation between age and proportion of active X chromosomes carrying the full mutation. *J Med Genet* 28: 830-836, 1991.
25. Brown W T, Houck G E, Jeziorowska A, Levinson F N, Ding X, Dopkin C, Zhong N, Henderson J, Brooks S S, Jenkins E C. Rapid fragile X carrier screening and prenatal diagnosis using a non radioactive PCR test. *JAMA* 270: 1569-75, 1993.
26. Eichler E E, Holden J J A, Popovich B W, Reiss A L, Snow K, Thibodeau S N, Richards C S, Ward P A, Nelson D L. Length of uninterrupted CGG repeats determines instability in the FMR 1 gene. *Nat Genet* 8: 88-94, 1994.
27. Das S, Kubota T, Song M, Daniel R, Berry-Kravis E M, Prior T W, Popovich B, Rosser L, Arinami T, Ledbetter D H. Methylation analysis of the fragile X syndrome by PCR. *Genetic Testing* 1(3): 151-5, 1998.
28. Levinson G, Maddalena A, Palmer FT, Harton GL, Bick DP, Howard-Peebles PN, Black SH, Schulman JD. Improved sizing of fragile-X CGG repeats by nested polymerase chain reaction. *Am J Med Genet* 51: 527-534, 1994.