

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

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Note: This supersedes Technical Standards and Guidelines for Fragile X Testing: The First of a Series of Disease-Specific Supplements to the Standards and Guidelines for Clinical Genetics Laboratories of the American College of Medical Genetics and Genomics (*Genetics in Medicine* 2001;3(3):200) and Technical 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 (2006).

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Molecular genetic testing of the *FMR1* gene is commonly performed in clinical laboratories. Mutations in the *FMR1* gene are associated with fragile X syndrome, fragile X tremor ataxia syndrome, and premature ovarian insufficiency. This document provides updated information regarding *FMR1* gene mutations, including prevalence, genotype–phenotype correlation, and mutation nomenclature. Methodological considerations are provided for Southern blot analysis and polymerase chain reaction amplification of the *FMR1* gene, includ-

ing triplet repeat–primed and methylation-specific polymerase chain reaction. In addition to report elements, examples of laboratory reports for various genotypes are also included.

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Key Words: *FMR1*; fragile X syndrome; Southern blot analysis; triplet repeat–primed PCR

The American College of Medical Genetics and Genomics Laboratory Quality Assurance Committee has the mission of maintaining high technical standards for the performance and interpretation of genetic tests. In part, this is accomplished by the publication of the document “ACMG Standards and Guidelines for Clinical Genetics Laboratories,” which was published in its second edition in 1999 and is now maintained online (http://www.acmg.net/StaticContent/SGs/Section_G_2010.pdf). This subcommittee also reviews the outcome of national proficiency testing in the genetics area and may choose to focus on specific diseases or methodologies in response to those results. Accordingly, the subcommittee selected fragile X syndrome to be the first topic in a series of supplemental sections, recognizing that it is one of the most frequently ordered genetic tests and

that it has many alternative methods with different strengths and weaknesses. This document is the second update to the original Standards and Guidelines for Fragile X testing that were published in 2001. It is designed for genetic testing professionals who are already familiar with the disease and the methods of analysis.

FX 1: INTRODUCTION

Disease-specific statements are intended to augment the current general *ACMG Standards and Guidelines for Clinical Genetics Laboratories*. Individual laboratories are responsible for meeting the Clinical Laboratory Improvement Amendments/ College of American Pathologists (CAP) quality assurance standards with respect to appropriate sample documentation, assay validation, general proficiency, and quality control measures.

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FX 2: BACKGROUND ON FRAGILE X SYNDROME**FX 2.1: Gene symbol/chromosome locus**

FMRI is the gene symbol recognized by the HUGO gene nomenclature committee (http://www.genenames.org/data/hgnc_data.php?hgnc_id=3775). The gene was referred to as *FXA* in the past. The chromosome locus is Xq27.3.

FX 2.2: OMIM number

The OMIM numbers are as follows: *309550 (*FMRI*), 300624 for fragile X mental retardation syndrome (includes fragile X-associated primary ovarian insufficiency), and 300623 for fragile X tremor/ataxia syndrome (FXTAS).

FX 2.3: Brief clinical description

The features of fragile X syndrome include specific cognitive deficits and certain characteristic, but nonspecific, physical features and behaviors. Most individuals with the premutation do not show fragile X syndrome-related features; however, some with high repeat sizes (>100 repeats) have been identified with learning difficulties, emotional problems, or even intellectual disability.¹ Females with premutations (usually >80 CGG repeats) are at ~20% risk for premature ovarian insufficiency (POI).²⁻⁷ This condition is now referred to as FXPOI. There is no evidence to support an association between high normal and intermediate range *FMRI* alleles with a risk of POI.^{8,9} Older males and females with premutations are at risk for FXTAS.¹⁰⁻¹⁶ FXTAS is a late-onset, progressive development of intention tremor and ataxia often accompanied by progressive cognitive and behavioral difficulties including memory loss, anxiety, reclusive behavior, deficits of executive function, and dementia. The risk for FXTAS is higher in males who carry a premutation as compared with females. The penetrance of FXTAS increases with age and with premutation repeat length.^{17,18} Guidelines to identify patients for whom FXTAS testing is indicated are available.¹⁹ For more information on these disorders, see the online GeneReviews profile for *FMRI*-related disorders at <http://www.ncbi.nlm.nih.gov/sites/GeneTests/> and the National Fragile X Foundation at <http://www.nfxf.org>.

FX 2.4: Mode of inheritance

Inheritance of the *FMRI* mutation is X-linked, although the pattern of fragile X syndrome is complicated due to the characteristics of the unstable repeat sequence mutation. In typical fragile X families, the mutation is a multistep expansion occurring over one or more generations in a region of CGG repeats in the 5' untranslated region of the gene. Small expansions (premutations) are not generally associated with cognitive deficits in males and females. Large expansions (full mutations) are penetrant in all males and many females. With extremely rare exceptions, the parent of origin of the expansion to the full mutation is female.

FX 2.5: Gene description/normal gene product

The gene product is fragile X mental retardation protein (FMRP), a widely expressed RNA-binding protein. The fragile

X syndrome is caused by a loss of the FMRP. FMRP is a selective RNA-binding protein that can form a messenger ribonucleo-protein complex that can associate with polysomes.²⁰ FMRP has been shown to behave *in vitro* as an inhibitor of protein translation.²¹ At the neuroanatomic level, the fragile X brain differs from normal brain due to the presence of unusually long and thin dendritic spines in the cortical regions.^{22,23} The dendritic spines are the location where excitatory synaptic transmission occurs. FMRP appears to be associated with polyribosomes within dendritic spines of “wild-type” neurons.²⁴ From these data, it has been proposed that FMRP is a translation suppressor that regulates protein synthesis locally in dendrites in response to synaptic stimulation signals.²⁵ In the fragile X brain, translation of certain messages may be exaggerated because the normal inhibition provided by FMRP is absent.²⁶

Studies of *FMRI* mRNA expression provide evidence that expansion in the premutation range perturbs gene expression and may have pathophysiological consequences, particularly those related to FXTAS and ovarian failure (see section 2.3). Reductions in the amount of FMRP have been found in both lymphocytes and transformed lymphocytes of premutation carriers.^{27,28} Using a highly sensitive fluorescent assay, Kenneson *et al.*²⁸ demonstrated a decrement in FMRP in individuals with expansions only slightly larger than the upper edge of the normal range. The reduction in FMRP is associated with an increase in *FMRI* mRNA^{1,27-29} in individuals with premutations. Expansion of the CGG repeats into the premutation range can shift transcription of *FMRI* mRNA from the usual, downstream-most start site to upstream sites. The utilization of alternative start sites may be correlated with increased *FMRI* mRNA transcription levels.³⁰ RNA-mediated toxicity may result in the FXTAS phenotype.³¹

FX 2.6: Mutational mechanism/abnormal gene product

Fragile X syndrome is caused by the deficiency or absence of FMRP. Theoretically, this can occur through any type of deletion or inactivating mutation, but in more than 99% of cases, there is an expansion of a segment of CGG repeats in the 5' untranslated region of *FMRI*. Large CGG expansions in this region are associated with hypermethylation and inhibition of transcription.

FX 2.7: Listing of mutations

Mutations at locations other than the CGG repeat have been described. A listing can be found in the Human Gene Mutation Database at <http://www.hgmd.cf.ac.uk/ac/index.php>. Guidelines for detecting these relatively rare mutations are beyond the scope of this document.

FX 2.8: Prevalence and ethnic association of common mutations

FX 2.8.1: Full mutations. The general prevalence of males with a full mutation is estimated as ~1 in 4,000. The female prevalence rate is presumed to be approximately one-half of the male rate, or ~1 in 5,000–8,000.³² Two large North American studies using

anonymized newborns identified a prevalence of 1 in 5,161 US males³³ and 1 in 6,209 Canadian males.³⁴

All major ethnic groups and races appear to be susceptible to expansion of the *FMR1* CGG region.^{32,35} An extensive literature review indicated a prevalence of fragile X syndrome ranging from 1 in 3,717 to 1 in 8,198 among Caucasian males in the general US population.³⁶ In another study carried out over 4 years in metropolitan Atlanta, the prevalence of fragile X syndrome was estimated to be 1 in 2,545 among African-American males and 1 in 3,717 among Caucasian males.³⁷ However, the prevalence estimate for Caucasian males, determined from this and from other studies, fell within the 95% confidence interval for African-American males. The prevalence of the fragile X mutation in an Afro-Caribbean population in the French West Indies was similar (1 in 2,539) to that in the African-American population in Atlanta.³⁸ Falik-Zaccari *et al.*³⁹ have suggested that the Tunisian Jewish population is the only other ethnic group to have a higher prevalence of the fragile X syndrome than the Caucasian population. However, these studies have not been supported by other data.⁴⁰ A blinded Taiwanese study estimated the prevalence of fragile X as 1 in 10,046 males.⁴¹

Further studies are required to determine if the frequency of the fragile X syndrome differs in ethnic populations.

FX 2.8.2: Premutations. A study of 2,300 US women identified 1 in 382 as carriers.⁴² In the largest US study to date (of over 119,000 tested), the overall carrier frequency among US females was 1.3% (0.61% for full mutation and 1.7% for premutation).⁴³

A recent large-scale study of 21,411 anonymous Canadian females (mothers of newborns) identified 1 in 549 as carriers.³⁴ Previous screens for the prevalence of premutations (with 55–101 repeats) in French-Canadian women estimated the carrier frequency to be 1 in 259.⁴⁴ A subsequent study revealed a 1 in 1,760 prevalence of premutation alleles among Canadian males.⁴⁵

A study from Israel of 36,483 women who requested screening identified 1 in 157 as carriers.⁴⁶ This is consistent with an earlier study of 9,459 women in Israel that found 1 in 152 with alleles having >54 repeats.⁴⁷ In the individuals with no family history of the fragile X syndrome, 1 in 166 women were determined to have premutations with a CGG-repeat range of 55–101. This estimate of the premutation carrier frequency is approximately twofold higher than that reported in the studies performed in Canada. Toledano-Alhadeff *et al.*⁴⁰ obtained similar values when studying 14,334 preconceptual or pregnant women in Israel, namely, 1 in 113 women with >54 CGG repeats. This study excluded women with a family history of mental retardation. In addition, they found that the premutation carriers were well distributed among all the Jewish ethnic groups, in contrast to a previous study.³⁹

An Italian study of ~2,000 mothers and their newborns in the general population found a premutation carrier frequency of 1 in 109 females and 1 in 225 newborn males (56–70 CGG repeats).⁴⁸ A fragile X screen of 10,000 newborn males in Taiwan showed a prevalence of 1 in 1,674.⁴¹ Therefore, the carrier

frequencies vary widely among populations and may be higher than those determined in the French-Canadian population.

Among females with POI and simplex cases of adult males with cerebellar ataxia, the *FMR1* premutation is identified in 4–6% and 2%, respectively.^{49,50}

FX 2.9: Special testing considerations

FX 2.9.1: Sensitivity and specificity. CGG-repeat-expansion full mutations account for >99% of cases of fragile X syndrome. Therefore, tests that effectively detect and measure the CGG-repeat region of the *FMR1* gene are >99% sensitive. Positive results are 100% specific. There are no known forms of FMRP deficiency that do not map to the *FMR1* gene. Fragile X syndrome should not be confused with the unrelated syndrome associated with the *FRAXE* locus.

FX 2.9.2: Indications for testing. The ACMG and American Congress of Obstetricians and Gynecologists have published recommendations regarding fragile X testing for diagnostic testing and carrier detection.^{51–53}

The identification of a full mutation in a male is considered diagnostic rather than predictive, inasmuch as penetrance of fragile X syndrome is virtually 100% in males and the age of onset is not variable.

The identification of a full mutation in a female may be diagnostic, but <50% of females with full mutations have intellectual disability. They may, however, have some manifestations of the disease such as avoidance personality, mood, or stereotypic disorders.^{54,55} Nonrandom X inactivation may explain the milder phenotype in females, although the extent of symptoms cannot be determined by X-inactivation patterns from diagnostic tests that determine the expansion and methylation in blood.

The identification of a premutation in an asymptomatic male or female undergoing carrier testing (e.g., due to a family history of intellectual disability) is predictive because FXPOI and FXTAS are not fully penetrant and are dependent on both age and allele size.

Population carrier screening and newborn screening for fragile X syndrome are not recommended at this time and should occur only under a research protocol.

FX 2.9.3: Prenatal testing. This test can be used for prenatal diagnosis in cells obtained from amniocentesis and chorionic villus sampling (CVS). Because methylation is not fully established at the time of CVS, the appearance of full mutations examined by a methylation-specific method may vary in CVS as compared with blood and amniocytes. Laboratories offering testing of chorionic villi must be aware of this tissue's unique properties:

- Methylation associated with lyonization is usually not present, and methylation associated with full mutations may or may not be present.⁵⁶ In the past, the hypomethylated status of this locus in this tissue had been thought of as a limitation or possible source of confusion. However, because it is unwarranted to use methylation status or

X-inactivation for phenotypic prediction of a full mutation, the possible hypomethylation of this tissue is no disadvantage, provided that the tissue-specific basis of the hypomethylation is understood.^{56,57} It is an acceptable option to omit methylation analysis entirely when testing CVS specimens. In the minor fraction of CVS cases with a result that is ambiguous between a large premutation and a small full mutation by size criteria alone, a follow-up amniocentesis may be required;

- The degree of somatic variation in a full mutation “smear” has a wider range of possibilities than is typically seen in blood specimens, from very limited to extraordinarily diffuse;
- Mosaicism between trophoblasts and somatic cells is theoretically possible. For this reason, when CVS results indicate a premutation, follow-up amniocentesis has been suggested to rule out mosaicism for a full mutation. However, there has been no known occurrence of this type of mosaicism.

FX 2.10: Nomenclature

The use of standard nomenclature is important for the accurate communication of results to health-care providers and is recommended by the ACMG⁵⁸ and CAP.⁵⁹ According to Human Genome Variation Society recommendations regarding the nomenclature for short sequence repeats, the designation for the *FMRI* triplet repeat (based on the coding DNA reference sequence, NM_002024.5) is c.-129CGG[X]. The start of the variable repeat is specified by -129, and CGG indicates the sequence of the repeat unit. The number of triplet repeats present is specified by X. If the exact size of the repeat cannot be determined (e.g., full mutations sized by Southern blot analysis), then the square brackets are replaced by parenthesis, (X), to signify uncertainties.⁶⁰ Current Human Genome Variation Society recommendations do not address nomenclature for nucleotide repeat mutations with size mosaicism.

Standard nomenclature is recommended, although laboratorians and clinicians may continue to use common mutation nomenclature. Therefore, to avoid confusion, it is acceptable to describe a mutation using standard nomenclature followed by the common name in parentheses or to use the common name in one section of the report (e.g., results) and the standard name in another section (e.g., description of methods or interpretation).

FX 3: GUIDELINES

FX 3.1: Definition of normal and mutation categories

There are four allelic forms of the gene: normal, intermediate, premutation, and full mutation. The associated number of CGGs for each can be defined on the basis of our current information to date. It must, however, be recognized that the borders of each definition may change with increased empirical data and research.

FX 3.1.1: Normal alleles. Normal alleles have a range of ~5 to ~44 repeats. The most common repeat length is 29 or 30 CGG repeats. Normal alleles have no meiotic or mitotic instability.

FX 3.1.1.1: In stable, normal alleles, the CGG region is interrupted by an AGG triplet after every 9 or 10 CGG repeats. The AGG triplets are thought to anchor the region during replication and prevent strand slippage. Direct testing for the AGG triplets is available clinically, although it is not routinely performed and its clinical usefulness is yet to be determined. It may help predict risk of expansions from premutations of <100 repeats.⁶¹

FX 3.1.2: Intermediate (gray zone, inconclusive, borderline).

The range from ~45 to ~54 repeats is intermediate (also referred to gray zone, inconclusive, or borderline). Alleles in this range can be considered normal in the sense that such alleles are not associated with fragile X syndrome and have not been observed to expand to a full mutation in one generation. Although earlier studies suggested an association between alleles in this size range and FXPOI, larger subsequent studies did not support these initial findings.^{8,9} A small number of patients meeting the criteria for FXTAS with *FMRI* intermediate alleles have been described,⁶² although larger studies are needed to determine the significance of this finding.

Minor increases and decreases in repeat number can occur when alleles of this size are passed on, but there is no measurable risk of a child with fragile X syndrome in the next generation. Alleles of this size may be associated with fragile X syndrome in future generations or in distant relatives. Alleles in this range can be referred to as premutations if they are confirmed by family studies to be traceable to a known full mutation or unambiguous premutation. A gray-zone allele of 52 repeats was reported to expand to a premutation allele of 56 repeats in one generation, which subsequently expanded to a full mutation allele in the next generation.⁶³ Testing at-risk relatives of individuals with an intermediate allele may determine the stability of the allele in the family. However, the rate of expansions of intermediate alleles is not well understood.

FX 3.1.3: Premutation. Premutation alleles range from ~55 to ~200 repeats. These alleles are long repeat tracks that are unstably transmitted from parent to child. Expansions from the premutation size range to the full mutation typically occur during maternal transmission. Due to the possibility of somatic mosaicism, careful examination for mosaicism into the full mutation range is recommended when a premutation is detected. *FMRI* alleles in the premutation size range are not hypermethylated and are not associated with fragile X syndrome. Although males and females with premutations and manifestations of some symptoms of fragile X syndrome have been reported, further studies are needed.⁶⁴ Women with alleles in this range are considered to be at risk for having affected children.⁶⁵⁻⁶⁷ The smallest *FMRI* premutation allele reported to expand to a full mutation in a single generation is 56 repeats.⁶³ Females who carry an *FMRI* premutation should be offered prenatal diagnosis for all pregnancies. Some clinicians may offer prenatal diagnosis to women whose reproductive partner carries a premutation as well. All at-risk family members of

known carriers should be offered testing to determine their status.

FX 3.1.3.1: The upper limit of premutations is sometimes said to be ~230. In fact, both numbers (200 and 230) are rough estimates derived from Southern blots in which large premutations were measured with increases of 0.5 to 0.6 kb, implying ~170 to 200 more triplet repeats than normal.

FX 3.1.4: Full mutations. Full mutations have more than 200–230 repeats, typically several hundred to several thousand repeats. There is usually broad somatic variation within each patient. Hypermethylation is typically present on most or all copies, with the exception of DNA extracted from CVS (see section 2.9.3).

FX 3.1.5: Mosaicism. Mosaicism due to *de novo* somatic mutations does not occur at the *FMRI* CGG-repeat region, but size mosaics and methylation mosaics have been observed.^{68–71} When mosaicism is present, tissue-specific differences can be seen. Individuals with size or methylation mosaicism may be higher functioning than individuals with completely methylated full mutations.

FX 3.1.5.1: Size mosaics. This term refers to an individual with subpopulations of full mutations, which are methylated, and premutations, which are unmethylated. Occasionally, there also may be minor subpopulations with near-normal or normal length. For this reason, care must be taken to examine for larger alleles when a normal or gray-zone allele is detected using standard PCR methods.

FX 3.1.5.2: Methylation mosaics. This term refers to individuals with an *FMRI* allele in the full mutation size range, with subpopulations of cells containing an unmethylated full mutation and other populations of cells containing a methylated full mutation.

FX 3.2: Methodological considerations

All general guidelines for Southern blot analysis and PCR in the *ACMG Standards and Guidelines for Clinical Genetics Laboratories* apply (<http://www.acmg.net>). The following additional details are specific for fragile X. For this test, there are many valid methods with different strengths and weaknesses. Laboratories will likely need to use more than one method because no single method can characterize all aspects of the *FMRI* full mutation, and precision in determining allele size varies between PCR and Southern blot analysis. For mosaic samples spanning the premutation and full mutation ranges, traditional PCR may amplify the premutation population but not the subpopulation with the full mutation. The expected phenotype for an individual with a premutation versus mosaicism for a premutation and full mutation is very different. Therefore, not detecting the full mutation would result in a different risk assessment for fragile X, FXTAS and FXPOI.⁵¹ For this reason, the ACMG policy statement recommends that Southern blot analysis always be performed along with traditional PCR, even if a premutation allele is identified by traditional PCR.

However, newer repeat-primed PCR methods or methylation PCR eliminate the need to perform Southern blot analysis on every sample (see section 3.2.2.10).

FX 3.2.1: Southern blot analysis

FX 3.2.1.1: Probe and restriction site combinations. **Table 1** describes several single- and double-enzyme options that are commonly used and several probes that are available.^{72–75} Other restriction enzymes and probes can be used, if equivalence is demonstrated. Probes can be sustained in plasmid DNA and isolated using a plasmid preparation procedure, they can be prepared by PCR amplification, or they can be purchased commercially.

FX 3.2.1.2: In general, when using the StB12.3 probe, small premutations are more easily detected when the normal fragment is small and/or electrophoretic migration is long, whereas large/diffuse full mutations are more easily detected when the normal fragment is large and/or electrophoretic migration is short.

FX 3.2.1.3: Controls should be included to confirm the proper choice and activity of restriction enzymes and probe. They should ideally represent the more difficult-to-recognize genotypes. To verify digestion and hybridization parameters, a normal control will suffice. However, in fragile X blot analyses, the abnormal controls are extremely important because they provide quality control on the resolution of small premutations and the detectability of diffuse smears. Characterized reference material possessing specific *FMRI* premutation CGG-repeat sizes may be obtained from the CDC Genetic Testing Reference Materials Coordination Program⁷⁶ through the Coriell Institute for Medical Research.⁷⁷

FX 3.2.1.4: For female patients, it should be noted that the degree of separation between two differently sized normal alleles could appear identical with that between a normal and a premutation allele (e.g., 20 and 44 repeats vs. 35 and 59 repeats). A Southern blot analysis with superior resolution and appropriate size standards or controls is required to distinguish between these possibilities (**Figure 1**). Alternatively, most PCR-based methods can provide the required resolution. Similar considerations apply to detection of premutation alleles in normal transmitting males.

FX 3.2.1.5: Because full mutations can be extremely diffuse and faint, signal to noise ratios must be very good. Laboratories are advised to be aware of the many different

Table 1 Probe and restriction site combinations

Primary restriction sites and normal length	Optional internal methyl-sensitive sites	Probes and references
<i>EcoRI</i> , 5.2 kb	<i>EagI</i> , <i>BssHI</i> , <i>NruI</i> , etc.	StB12.3 (ref. 72), pE5.1 (ref. 73)
<i>HindIII</i> , 5.4 kb	Ox1.9 (ref. 74)	<i>BglII</i> , 12 kb
<i>PstI</i> , 1.0 kb	NA	pfxa3 (ref. 75), Ox0.55 (ref. 74)

NA, not appropriate.

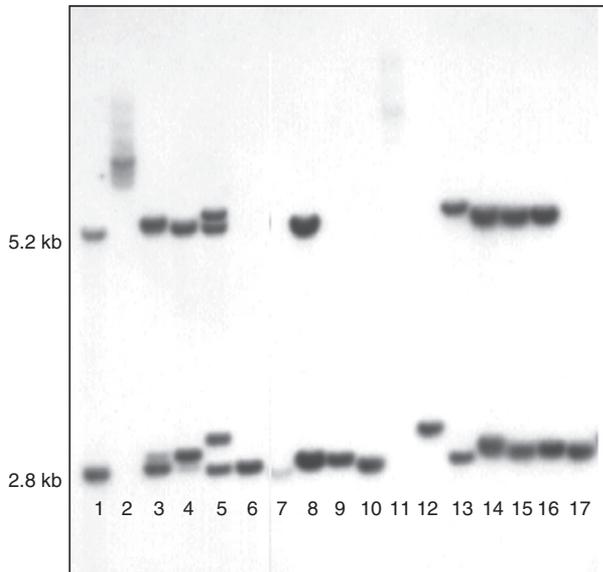


Figure 1 Southern blot using *EcoRI* and *EagI* digestion, probed with StB12.3, using extended electrophoresis to illustrate several subtle specimen types.

1: Normal female; 2: full-mutation male, note the combination of a predominant band with a diffuse smear; 3: female with 28 and 52 repeats, with the smaller allele predominantly active; 4: female with 26 and 52 repeats, with the larger allele predominantly active; 5: female with 18 and ~80 repeats, with equal X-inactivation; 6: normal male; 7: normal male, underloaded and smiling due to DNA degradation (the apparent line between lanes 6 and 7 is a photographic artifact); 8: normal female; 9: normal male; 10: normal male; 11: affected male, underloaded and very diffuse; 12: premutation male; 13: female with 20 and 70 repeats, with the smaller allele virtually exclusively active (the only evidence of abnormality is the slow migration of the “5.2 kb” band); 14: female with 27 and 42 repeats, with the larger allele somewhat more active; 15–17: unremarkable normal females and male. Figure provided by Genetics & IVF Institute. Reprinted from ref. 101.

appearances of full mutations. Full mutations are not likely to be overlooked in males, inasmuch as the normal signal will be absent (or light, in size mosaics), but full mutations can be easily missed in females if the background is poor. Skewed X-inactivation may also present problems in the use of Southern blot analyses performed with methylation-sensitive restriction enzymes in the detection of females with premutations or full mutations.

FX 3.2.1.6: Migration distances should be interpreted using a standard ladder such as lambda *Hind* III fragments or a set of carefully chosen, independently tested human references.

FX 3.2.1.7: The following guidelines refer to methylation analysis using two different restriction enzymes, one of which is methylation sensitive.

FX 3.2.1.7.1: In DNA extracted from tissues other than chorionic villi, methylation analysis reveals the degree of hypermethylation in full mutations and shows the distribution of X-inactivation in any female with two distinguishable alleles. Southern blot analysis with the addition of methylation-sensitive enzyme digestion can:

- Help discriminate between premutations and full mutations for the rare alleles that fall near the boundary (i.e., around 200 repeats); and
- Detect rare individuals who are methylation mosaics.

FX 3.2.1.7.2: In DNA extracted from tissues other than chorionic villi, the results of routine methylation analysis and PCR are sometimes confounded by an abnormal karyotype such as 45,X or 47,XXY. Individuals with testicular feminization (XY females) will have a male methylation pattern. In these cases, gender should be confirmed. Interpretation of results should take the individual’s karyotype into account, when it is available to the molecular laboratory.

FX 3.2.1.7.3: In DNA extracted from tissues other than chorionic villi, methylation analysis increases the difficulty of detecting females with small premutations who have highly skewed X-inactivation. Double digestion with a methylation-sensitive restriction enzyme causes the signal from each allele in a female to be split into active and inactive bands, forming four bands in a carrier female. When X-inactivation is balanced in a carrier, the two active bands are readily seen, although the two inactive bands may comigrate. However, if X-inactivation is heavily skewed, there will be only two visible bands. This is particularly challenging when the premutation is predominantly inactive because then it appears only in the upper region of the gel, where resolution is considerably poorer. For an example of a carrier with extremely skewed X-inactivation, see lane 13 of **Figure 1**. Lanes 3 and 4 show two females with oppositely skewed X-inactivation. The above data are true for the use of the StB 12.1 probe. Use of other probes such as pE5.1 will yield an additional small control band.

FX 3.2.1.7.4: *FMR1* methylation status should not be used to predict severity in fetal or newborn cases, regardless of whether the DNA was extracted from amniocytes, chorionic villi, or blood.

FX 3.2.1.7.5: In DNA extracted from chorionic villi, the *FMR1* region usually does not have methylation associated with X-inactivation, and it may or may not have hypermethylation associated with full mutations if the CVS procedure was performed before 12.5 weeks gestation.⁵⁶ When testing DNA extracted from chorionic villi, methylation analysis is optional. Incidentally, methylation analysis before 12.5 weeks gestation can serendipitously alert a laboratory to maternal cell contamination in chorionic villus specimens inasmuch as methylation associated with X-inactivation is usually not present at this locus in tissue obtained via CVS; a strong normal inactive band can be a sign of possible maternal cell contamination. Other explanations for such a band include X-inactivation in some fetal cells or incomplete digestion. Further investigation would be called for.

FX 3.2.2: PCR methods

FX 3.2.2.1: Several sets of primers, PCR conditions, and methods of separation and detection have been published.^{78–81} Other primers and methods can be used if equivalence is

demonstrated. A particular region to be aware of in primer design is the deletional hotspot.⁸² PCR can be performed using ³²P and electrophoresis on an acrylamide gel, or more commonly incorporating a fluorescently labeled primer followed by capillary electrophoresis (CE).

FX 3.2.2.2: Regardless of the locus, any PCR can theoretically fail to detect an allele if there is a polymorphism at the primer-binding site. There are no known polymorphisms that would affect any of the commonly used primers.

FX 3.2.2.3: Patient amplicon sizes should be determined using a size standard. For CE, a standard fluorescent-labeled size marker can be used. For acrylamide gels, a standard ladder, such as an M13 sequencing reaction or a set of carefully chosen, independently tested human references, can be used.

FX 3.2.2.4: Controls representing the genotypes to be distinguished should be used for each run. Refer to section 3.2.1.3 for a discussion of reference materials. The upper limit of allele size that can be successfully detected should be known, and a control corresponding to that size should be included in each run. Laboratories should confirm the size of their control DNA by sequencing (if possible) or by using verified reference materials.

FX 3.2.2.5: Amplification of CG-rich regions is difficult, and special conditions are required. The difficulty increases with increasing numbers of CGG repeats; therefore, many PCR strategies do not attempt to detect large alleles. In such a system, it is not possible to tell the difference between a female who is homozygous for a normal allele and one who has a large nonamplifiable second allele. Similarly, patients who are mosaics for premutations and full mutations will appear to have only premutations.

FX 3.2.2.6: When a PCR strategy is capable of detecting large alleles, amplification nevertheless may favor the smaller allele in any specimen with multiple alleles, i.e., females and mosaics.

Such methods should be validated with carrier females and mosaics, in addition to males. Because of disproportionate amplification, PCR is not reliable for determining the ratio of different species in a mosaic individual.

FX 3.2.2.7: In PCR amplification of samples from females and mosaics, heteroduplexes can form. If denaturing polyacrylamide gel electrophoresis is used, conditions must be sufficiently denaturing to avoid heteroduplex artifact. If nondenaturing polyacrylamide gel electrophoresis is used, steps must be taken to distinguish between heteroduplexes and true abnormal alleles.

FX 3.2.2.8: Basic PCR amplification is not affected by methylation. Although PCR tests specifically modified to detect methylation status have been described,^{83,84} the original PCR strategies that have been in use for many years are completely independent of methylation.

FX 3.2.2.9: When a PCR strategy is used to detect full mutations, the presence of a deletion hotspot in the CGG-repeat region should be noted.⁸² Primers located within the deletion hotspot may result in failure to detect the expanded allele. Primers located upstream of the deletion hotspot may result in apparent size mosaicism.

FX 3.2.2.10: Triplet repeat–primed PCR

FX 3.2.2.10.1: Triplet repeat–primed PCR (TRP PCR) allows rapid detection of PCR products formed by a chimeric primer binding inside a triplet-repeat region. In TRP PCR for fragile X, one primer is anchored completely outside of the CGG-repeat region, whereas the other overlaps the CGG repeat and the adjacent nonrepeated sequence. A third primer can be anchored outside of the CGG region that, when paired with the opposite anchored primer, will amplify “over” the CGG repeat. This will increase the amount of full-length product from the largest CGG-repeat allele and in some assays enables accurate sizing of alleles up to 200 CGG repeats. From the chimeric primer annealing at each CGG repeat, multiple amplicons are made, forming products each with a length differing by three bases. A number of reports have been published describing TRP PCR for fragile X.^{85–89}

FX 3.2.2.10.2: Although products can be separated by ethidium-stained agarose gels to detect “smears,” combining TRP PCR with single-base-resolution fragment analysis, the “smear” on a lower-resolution agarose gel becomes characteristic “stutters” or “ladders” that are easily visualized. The stuttering will end at the allele with the greatest number of CGG repeats. An increase of this product can be seen, particularly if the third primer is used. For alleles with >200 repeats, a “compression” product can be seen, which can be used as a marker for an allele with >200 repeats, although the fragment cannot be sized (**Figure 2**). If a third primer is used, full mutations appear as a compression (or compacted) product. These are seen by CE as a compacted product of ~200 CGG repeats, thereby indicating a full mutation (**Figure 3**).

FX 3.2.2.10.3: PCR followed by CE at a single-base resolution has a high analytical sensitivity and specificity for detecting expanded alleles. A threshold can be set to distinguish premutation and full mutation (and intermediate, if desired) alleles from normal alleles. If no expansion is detected, no further testing is necessary. Alleles with a stuttering pattern past a threshold and consistent with an expansion can be tested further to determine methylation (by Southern blot analysis or methylation-sensitive TRP PCR) or determine size by traditional PCR and/or Southern blot analysis. Therefore, the simple yes/no answer for the presence of expansions can eliminate the need for Southern blot analysis in samples with a normal-sized and intermediate-sized *FMR1* allele(s).

FX 3.2.2.10.4: TRP PCR resolves the challenges associated with apparent homozygous females, because the normal allele will not outcompete the expanded allele.

The increased sensitivity of the TRP PCR assay also resolves the difficulty of detecting mosaic males because mosaicism can be detected to ~10%. Laboratories using TRP PCR are encouraged to define the sensitivity of their assay using DNA with a normal *FMR1* allele titrated with serial dilutions of full-mutation *FMR1* DNA.

FX 3.2.2.10.5: Methylation PCR methods. Several methods besides Southern blot analysis have been described to determine methylation. Methylation-specific PCR involves

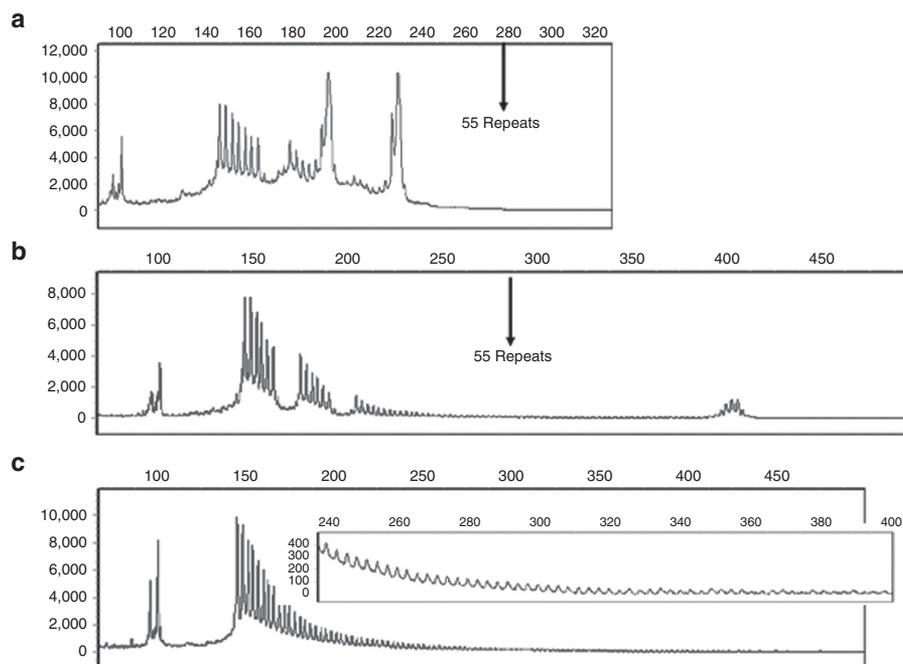


Figure 2 TRP PCR using a two-primer system. (a) Female with 20 and 31 CGG repeats. (b) Male with 103 CGG repeats. (c) Male with size mosaicism from ~140 to 800 CGG repeats (inset: reduced y-axis to better visualize baseline). TRP PCR, triplet repeat–primed PCR.

the differential treatment of DNA with methylation-specific restriction enzymes followed by allele-specific PCR and resolution of the PCR products with CE.^{90,91} This method not only determines methylation status but also *FMR1* allele size up to 250 repeats. However, to accurately size alleles >250 repeats, Southern blot analysis is needed.

FX 3.2.2.11: Other methods. Multiplexed ligation probe amplification has been described to identify males with methylated fragile X alleles.⁹² In this method, sequence-specific probes are hybridized to methylated and unmethylated alleles. Probes are simultaneously ligated and digested with a methylation-sensitive restriction endonuclease. A universal PCR primer set will amplify only probes that are ligated and undigested, indicating methylated alleles.

Real-time PCR has also been described with TaqMan chemistry and by melt-curve analysis, using methylation-specific PCR. TaqMan chemistry amplifies separately methylated and unmethylated specific alleles and provides a ratio based on amplification cycle thresholds.³³ Using melt analysis, methylated and unmethylated alleles are amplified simultaneously but due to differences of GC content can be resolved by differences in melting temperature between methylated and unmethylated alleles.⁹³ These methods have high analytical sensitivity and specificity for detecting methylation in males but are less sensitive and specific in females.

FX 3.3: Interpretations

FX 3.3.1: Example reports are included in the **Supplementary Appendix** online. In addition to the items described in the current general *ACMG Standards and Guidelines for Clinical Genetics Laboratories* (<http://www.acmg.net/StaticContent/>

SGs/Section_G_2010.pdf), the following elements should be included in the report:

FX 3.3.1.1: State whether the method used was PCR, Southern blot analysis, or both. If Southern blot analysis, state the restriction enzymes and probes used. If PCR, describe the PCR method used (e.g., TRP PCR) and method used for separation and detection (e.g., CE).

FX 3.3.1.2: State the definitions used for normal, intermediate (gray-zone, borderline, inconclusive), premutation, and full mutation.

FX 3.3.1.2.1: Note that it is not necessarily obvious that the borderline category refers to the border between normal and premutation and not to the border between premutation and full mutation. Similarly, note that the term *instability*, which is often used with regard to borderline alleles to describe minor inter-generational or mitotic changes, may unintentionally suggest a risk of having an affected child or personal late-onset symptoms.

FX 3.3.1.3: Classify the patient's result using the defined categories and Human Genome Variation Society nomenclature (refer to section 2.9.4). Common nomenclature can be included for clarity. The term *size mosaic* should be used for alleles that have significant subpopulations in both the premutation and full mutation range.

FX 3.3.1.4: All positive results should state that genetic counseling is recommended and testing is available for at-risk family members.

FX 3.3.2: The following descriptive elements may appear, with caution:

FX 3.3.2.1: The size of the alleles may be reported and could be of clinical use for premutation carriers. The premutation allele

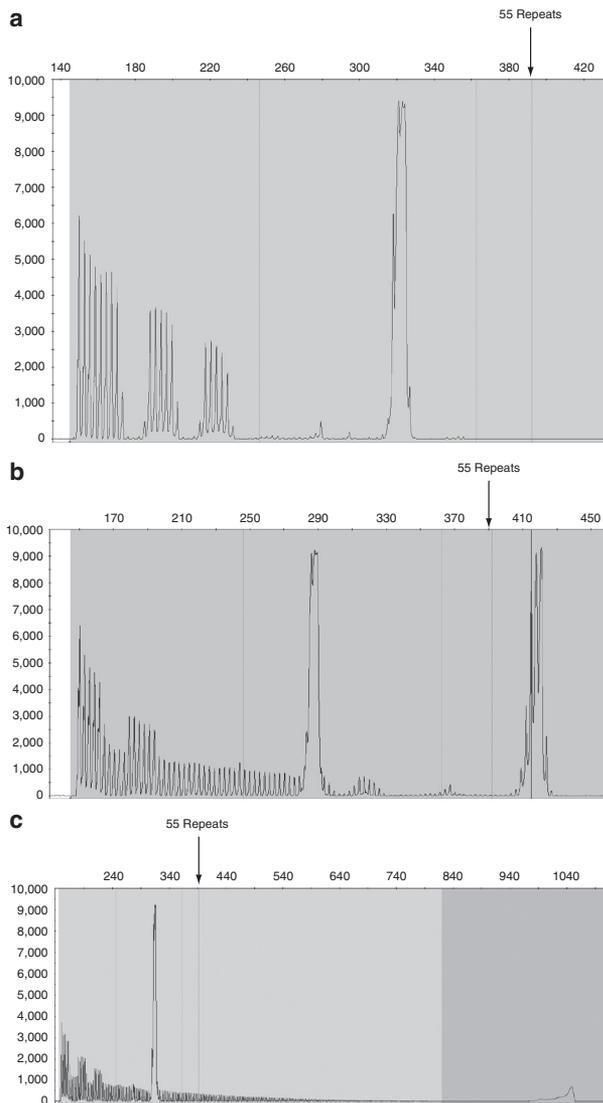


Figure 3 TRP PCR using a three-primer system. (a) Male with 32 CCG repeats. (b) Female with 20 and 64 CCG repeats. (c) Female with 29 and >200 CCG repeats. TRP PCR, triplet repeat–primed PCR.

size may be used for risk assessment in determining the chance of expansion in the offspring of carriers and also in determining the chance of FXTAS or FXPOI. If so, the precision used in quoting the size must be supportable by the precision of the size marker used, the sharpness of the bands or peaks, degree of stutter, and so on. It may be appropriate to state a range or use qualifying terms such as “approximately.” Descriptions such as “positive for an allele with 55–200 repeats” are ambiguous and should not be included in a laboratory report.

It has been the intent of the CAP/ACMG Biochemical and Molecular Genetics Resource Committee to standardize the accuracy of CGG-repeat quantitation. The acceptable range for sizing CGG repeats is based on the analysis of CAP/ACMG proficiency testing survey results for fragile X. Acknowledging the technical limitations of size analysis, the ACMG supports the following acceptable ranges for FMR1 clinical testing and/or as

grading criteria for the CAP/ACMG proficiency testing survey: consensus size ± 5 repeats for alleles with <55 repeats; consensus size ± 10 repeats for alleles with 56–100 repeats; and consensus size ± 2 SDs for alleles with >100 repeats.

FX 3.3.2.2: Description of methylation may be provided. The two kinds of methylation must be clearly distinguished: methylation due to X-inactivation and hypermethylation of full mutations. The term *methylation mosaic* or *incomplete methylation* may be used if not all molecules in a full mutation are hypermethylated.

FX 3.3.3: The following helpful points on alternative diagnoses may be included:

FX 3.3.3.1: There are rare forms of FMRP deficiency not caused by CGG expansion, which may not be detected by this test.

FX 3.3.3.2: Intellectual disability associated with other fragile X sites, in particular FRAXE, or other gene mutations will not be detected with this test.

FX 3.3.3.3: DNA analysis for fragile X syndrome should be performed as part of a comprehensive genetic evaluation that includes routine cytogenetic analysis⁵¹ and more recently chromosomal microarray analysis as recommended by ACMG.^{94,95}

FX 3.3.4: Comments on phenotype, if included, should be abstract rather than case specific. The following concepts apply:

FX 3.3.4.1: All males with full mutations have fragile X syndrome to some degree. The severity cannot be predicted from the size of the full mutation, but if premutations are also present or if the majority of the full-mutation molecules are unmethylated, the phenotype may be less severe.

FX 3.3.4.2: Females with full mutations exhibit a wide spectrum of phenotypes. They may be as severely affected as a male with an expanded fragile X allele (which is itself a range of phenotypes). Females with full mutations may also exhibit very mild learning disabilities or have no detectable deficits. The severity cannot be predicted from the size of the full mutation, nor can it be predicted from the pattern of X-inactivation.

FX 3.3.4.3: Individuals with premutations should not be interpreted as unaffected carriers. Females who carry a premutation are at risk for FXPOI and FXTAS. Males with the premutation are at risk for FXTAS. If an individual referred for diagnostic testing due to intellectual disability, autism, or learning disability is found to carry a premutation, no association can be stated at this point in time. It should be considered as a coincident finding unless FMRP deficiency or mosaicism for a full mutation is detected.

FX 3.3.4.4: Individuals with intermediate alleles should be interpreted as unaffected. Even more so than a premutation, an intermediate allele is considered a coincidence when found in an individual referred for diagnostic testing due to intellectual disability, learning disability, or autism. FMRP deficiency or mosaicism for a full mutation can be investigated by methylation-sensitive Southern blot analysis but with less

likelihood of success because intermediate alleles are common in the general population.

FX 3.3.5: Comments on reproductive risk, if included, should be abstract rather than case specific. The following concepts apply:

FX 3.3.5.1: All affected males and the overwhelming majority of affected females inherit their mutations from their mothers. The mothers carry either a premutation or full mutation.

FX 3.3.5.2: Women with full mutations have a theoretical 50% chance of passing on the full mutation with each pregnancy.

FX 3.3.5.3: Women with premutations have a theoretical 50% chance of passing on the fragile X mutation with each pregnancy. If it is passed on, the chance the allele will increase to a full mutation depends on its size in the mother. Probabilities range from 3% for maternal alleles with CGG repeats from 55 to 59 (1/23 transmissions) to ~100% for maternal alleles with 90 CGGs and above.⁶⁷ The smallest allele known to expand to the full mutation is 56 repeats.⁶³ Laboratories should be familiar with publications on this topic,^{65–67,78,96} including any current publications.

FX 3.3.5.4: Men with premutations will almost always pass premutations to all of their daughters. An extremely rare phenomenon involves unaffected males with premutations who have had affected daughters, apparently by gonadal mosaicism for full mutations.^{97–99} The sons of men with premutations are not at risk for developing the fragile X syndrome or FXTAS.

FX 3.3.5.5: To date, there have been no reports of male or female carriers of intermediate alleles having offspring with an FMR1 allele in the full mutation range. Instability may be identified if the allele can be traced through the family to a known full mutation or unambiguous premutation. In the absence of such a connection, it may be possible to show meiotic instability or a specific repeat sequence pattern (absence of AGG interruptions) that is at higher risk for instability. Testing for AGG status is available, although the clinical usefulness of this information is not known at this time.

FX 4: ALTERNATIVE TESTING METHODS

FX 4.1: Cytogenetic analysis

Testing for the fragile site FRAXA at Xq27 is no longer an acceptable diagnostic method. Clinical and analytical specificity and sensitivity are both insufficient.

FX 4.2: Protein analysis

Immunohistochemical staining for FMRP is a valid diagnostic method in lymphocytes.¹⁰⁰ Willemsen *et al.*⁵⁶ demonstrated that staining for the FMRP protein in chorionic villus samples could be used as an alternative prenatal diagnostic method for detection of full mutations in male fetuses. The situation is more complicated in female fetuses for which some chorionic villi may be completely positive and others from the same sample may be completely negative for FMRP staining. The authors' data shed light on the timing of X-inactivation in chorionic villus cells of the female fetus. The diagnostic application of this

method is not recommended at this time for the prenatal diagnosis of females carrying *FMR1* full mutations.

FX 5: POLICY STATEMENTS

FX 5.1

The American College of Medical Genetics and Genomics issued a policy statement titled "Fragile X Syndrome: Diagnosis and Carrier Testing" in 1994 (*Am J Med Genet* 53:380–381), which was updated in October 2005 (*Genet Med* 7:584–587). This document is also available online at <http://www.acmg.net/resources/policy-list.asp>. These Standards and Guidelines are in general agreement with that statement.

FX 5.2

In 2010, the American College of Obstetricians and Gynecologists issued a Committee Opinion, No. 469, on carrier screening for fragile X syndrome. These Standards and Guidelines are in general agreement with that opinion.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/gim>

DISCLOSURE

The authors direct laboratories that offer clinical molecular genetic testing for fragile X syndrome.

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