



The European Molecular Genetics Quality Network

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Draft Best Practice Guidelines for Molecular Analysis in Fragile X Syndrome

Draft guidelines prepared by Valérie Biancalana, Peter Steinbach and Su Stenhouse following discussions at the EMQN workshop, 23-24 November 2001, Strasbourg, France. **These guidelines have been fully updated and were published in January 2006**

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Disclaimer

These Guidelines are based, in most cases, on the reports drawn up by the chairs of the disease-based workshops run by EMQN and the CMGS. These workshops are generally convened to address specific technical or interpretative problems identified by the QA scheme. In many cases, the authors have gone to considerable trouble to collate useful data and references to supplement their reports. However, the Guidelines are not, and were never intended to be, a complete primer or "how-to" guide for molecular genetic diagnosis of these disorders. The information provided on these pages is intended for chapter authors, QA committee members and other interested persons. All the guidelines are at a draft stage, and must not be used until formally published. Neither the Editor, the European Molecular Genetics Quality Network, the Clinical Molecular Genetics Society, the UK Molecular Genetics EQA Steering Committee nor the British Society for Human Genetics assumes any responsibility for the accuracy of, or for errors or omissions in, these Guidelines.

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NOMENCLATURE AND GENE ID

See table 1

Locus	FRAXA	Gene	FMR-1
OMIM#	309550	GeneCard	Fragile-X

DESCRIPTION OF THE DISEASE

The fragile X syndrome (a.k.a. Martin-Bell Syndrome) is the most common cause of inherited mental retardation with an estimated incidence of 1 in 4000-9000 males ¹ and 1 in 7000-15000 females. In affected boys, delay in language acquisition and/or behavioural problems are often the presenting symptoms. In addition to cognitive deficits, the phenotype of fragile X syndrome includes mild dysmorphic features (large everted ears, coarse facies, elongated face) and macroorchidism established around puberty. Behavioural disturbances including attention-deficit hyperactivity or autistic-like behaviour can be observed.

Females can also be affected. Approximately 50-60% of female carriers of the disease causing mutation will have mild to moderate mental retardation.

THE GENE AND THE MUTATIONS

The gene involved (termed FMR1 for Fragile X mental retardation), located in Xq27.3, encodes an RNA-binding protein (FMRP) ^{2,3,4}. FMRP has been shown to bind with high affinity to RNA structures called G quartets, and lists of candidate mRNA targets for FMRP action were defined. Proteins interacting with FMRP have been identified, two of which provide a link with the Rho/Rac signalling pathway implicated in other forms of X linked mental retardation ^{5,6,7,8}.

Fragile X syndrome is caused in most of cases by expansions of a (CGG) trinucleotide repeat in the 5'UTR of the FMR1 gene, and subsequent abnormal methylation of neighbouring CpG island leading to the loss of the protein product FMRP (Figure 1).

Figure 1:

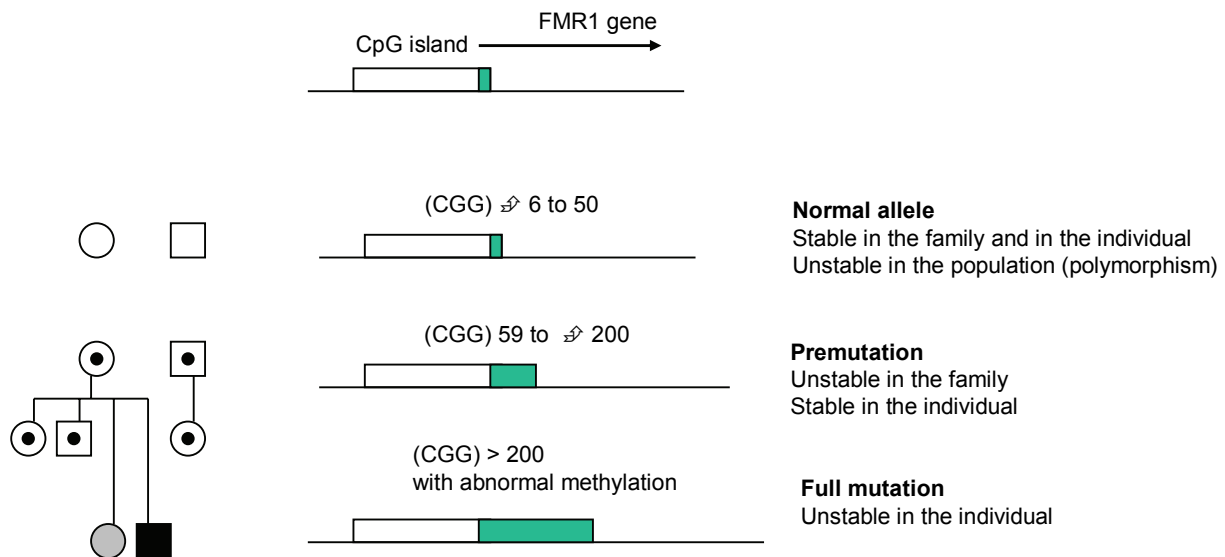


Figure 1 : Scheme of normal and expanded alleles at the FMR1 locus. On the top is the map of the DNA fragment that contains the CGG repeat at the 5' end of FMR1 gene (green box). The CpG region corresponds to a regulatory region that becomes abnormally methylated in the full mutation. On the left the genotype- phenotype correlation is indicated, with the pattern of transmission of expanded alleles. Empty symbols : normal allele, no mental retardation. Symbol with central dot : premutation carrier, with no mental retardation. Filled black symbol : full mutation carrier with mental retardation, filled grey symbol : full mutation carrier with mental retardation in 50-60% of the cases.

The normal form of the CGG repeat is polymorphic and contains from 6 to about 50 trinucleotide repeats, in general interspersed with 1-3 AGG triplets^{3,9,10} with 29-30 repeats being the most common alleles.

Alleles in the 59-200 CGG repeat range without abnormal methylation are referred to as premutations. Premutations do not cause mental retardation but were shown to be associated with premature ovarian failure in females and late onset tremor/ataxia syndrome in males^{11,12}. Some recent studies report the occurrence of an autistic disorder in patients with fragile X premutation, but this association deserves further study to estimate its prevalence^{13,14,15}. Premutations are unstable and, when transmitted by a female, have a risk of expanding to a full mutation. This risk of transition is very strongly dependent on the size of the maternal premutation, and is above 95% for alleles with more than 100 repeats^{16,17}. The smallest described allele that underwent transition to a full mutation in a single generation is 59 CGG repeats without AGG interruption¹⁸.

Alleles with CGG repeats in the intermediate range between normal and premutation (50 to 58 repeats) can show some instability upon paternal and maternal transmission. It is thought that 'pure' CGG repeats are less stable than repeats with interspersed AGG sequences¹⁹. The results from several epidemiological studies suggest that alleles above 52±1 CGG have a prevalence around 1 in 100 females in the general population (reviewed in²⁰).

The disease-causing full mutation is a large expansion exceeding 200 repeats, and is usually accompanied by methylation of the adjacent promoter region²¹ which results in transcriptional silencing of FMR1. Indeed, it appears that fragile X syndrome is due to absence of functional FMRP as deletions and point mutations in the FMR1 coding sequence have also been reported to cause the syndrome^{22,23,24,25}. These events are rare. A hot spot for deletions that occurred in the context of an expanded allele, has been identified in FMR1 exon 1^{26,27}.

Full mutations are unstable not only during transmission but also within fragile X patients. This somatic instability leads to two types of size mosaicism. (1) There is the wide range of repeat lengths of the full mutation. (2) Some rare fragile X patients are mosaic for a full mutation and an allele in normal range (or full mutation, premutation and normal range).

The term "Fragile X syndrome" is commonly used to refer to the syndrome caused by CGG expansions and other FMR1 mutations. However, in a small number of families, another fragile site has been linked to expansions at the nearby FRAXE locus of the FMR2 gene. FMR2 contains an unstable (CCG)_n repeat whose expansions are responsible for a milder phenotype of nonsyndromic mental impairment^{28,29,30,31}.

REASONS FOR REFERRAL

Reasons for referral are diffuse and include a wide range of mental impairment from profound mental retardation to mild learning difficulties, as well as developmental delay, autistic-like behaviours, hyperactivity and other behavioural problems..

Carrier detection and prenatal diagnosis are other reasons for referral.

Another indication is the occurrence of premature ovarian failure (POF) in a female, especially in cases of familial premature ovarian failure. Based on studies reviewed by S.L. Sherman¹¹, approximately 20% of female premutation carriers have POF compared to only 1% in the general population.

A possible indication is presenile tremor/ataxia (termed FXTAS) which might turn out to be associated with the fragile X premutation, particularly in males, but more studies are still warranted to establish its prevalence .

APPROACHES

The diagnostic procedure is mainly based on Southern blotting (DNA digested with specific restriction endonucleases) or on direct amplification of the CGG-repeat using flanking primers. Alternative immunocytochemical tests have been described.

Southern blotting allows the identification of all expansions as well as the determination of the methylation status. Using standard PCR amplification, alleles above 120 CGGs are difficult to detect although more efficient techniques are being developed. Accurate sizing of premutations requires the use of PCR. This is crucial to assess the risk of carrier females having affected children, and to distinguish intermediate alleles from premutations.

Attention is drawn in these guidelines to the potential for misinterpretation due to the specific pitfalls of each method, and to the technical limitation of each protocol. A statement of these limitations should appear clearly in the conclusion section of the molecular diagnosis report.

Southern blot analysis

This test is based on the sizing of the CGG repeat by Southern blotting. Southern blot analysis using a single enzyme (*EcoRI* with StB12.3 or equivalent probes, see figure 2) allows the detection of full mutations and large premutations, and is sufficient for testing of probands with learning difficulties. However, it is often combined with methylation analysis using a methylation-sensitive enzyme such as *EagI* or *NruI* which offers a very good strategy in family studies to detect premutations, full mutations and mosaic patterns³².

When high quality Southern blots with appropriate controls are analysed, interpretation is straightforward . However, high background and weak signal can lead to misinterpretations and, particularly, smears of expanded fragments can be very faint and easily overlooked.

The need for radioactive handling can be avoided since a non radioactive approach using a chemiluminescent probe has been tested ³³ and is currently used in several laboratories. High quality Southern blots were presented at the Strasbourg Best Practice Meeting by Wout Deleen (Rotterdam, The Netherlands), Christalena Sofocleous (Athens, Greece) and Paula Jorge (Porto, Portugal).

Figure 2:

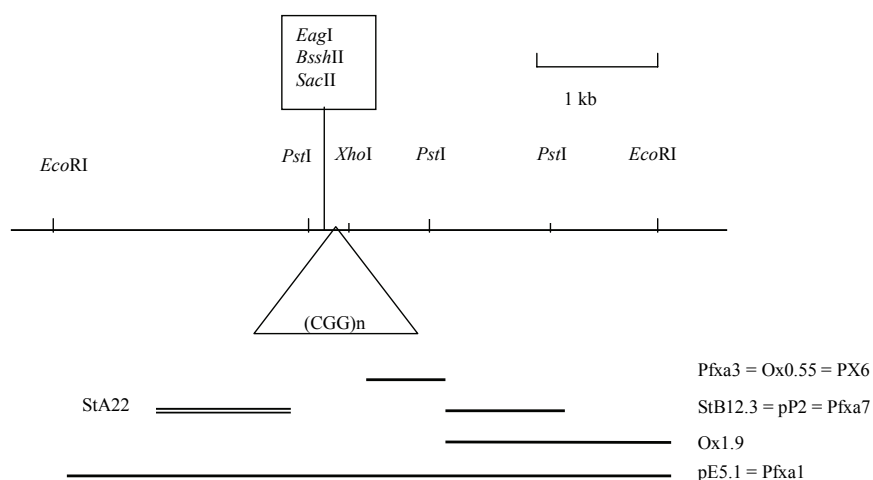


Figure 2 : Probes used in the diagnosis of Fragile X syndrome (modified from Oostra et al.⁶⁹)
 Probe Ox1.9 and Ox0.55 can be obtained from Professor Kay Davies in Oxford. St probes are available from Professor Jean -Louis Mandel in Strasbourg. Pfxa3 is available from the ATCC, with restrictions

PCR analysis

Compared to Southern blotting, the PCR test is more rapid and easy to use but its approach encounters particular technical difficulties ³⁴. Also, the primers should be checked carefully that they do not bind to a sequence where a SNP has been found. The amplification of large CG rich fragments is a laborious task and, since the first PCR test described by Fu et al.³, several methods have been published which detect alleles in the normal range and more or less in the premutation range (for example up to 200 repeats with Ethidium – visible detection ³⁵, and up to 120 repeats with automated capillary electrophoresis ³⁶). As a consequence, many laboratories use PCR as a first test, and only proceed to Southern blot on samples which fail to amplify (males) or show a single normal allele (homozygous normal females representing around 20% of females).

An internal control is required to distinguish a non amplifiable allele with a probable expansion from an unsuccessful PCR reaction³⁷. PCR for FMR1 can usefully be duplexed with PCR for FMR2²⁹ which provides an internal control with a similar CGG repeat but this strategy should be discussed with local clinicians before use. Some primer positions are indicated in Table 4.

One possible concern about using PCR for fragile X diagnosis is that affected males mosaic for normal and full mutations might be missed (MoMN individuals), as the PCR would only amplify the normal allele. In a study of 300 males with a full mutation ascertained by Southern blot and, using primers from Fu et al. 1991³, we have estimated that around 1% of full mutation males have a PCR amplifiable allele within the normal range. We have also detected a mosaic carrier female with 35 CGG, 50 CGG, premutated and full mutated alleles. Such cases would be diagnosed as normal using a PCR test. The possibility of size mosaics (normal/premutation or normal/full mutation) should therefore be taken into account, especially when testing in known fragile X families.

A false positive case has also been reported and shown to be caused by a 5 bp deletion preventing primer annealing and leading to an apparent null allele while the CGG repeat was in the normal range³⁸. This underlines the need for complementing PCR analysis with Southern blotting.

Nevertheless PCR tests have been described which should detect full mutated / normal allele mosaicism and are discussed below^{30,39,40,41,42}.

Immunohistochemistry FMRP protein

Test for the presence or absence of the FMRP protein have been developed using immunohistochemistry⁴³. Validated postnatal applications include blood smears for males and hair roots for both sexes. Logistic considerations are the major reasons for not making widely use of the FMRP test in diagnostic laboratories. Application on blood smears requires either fresh ('same day') blood or needs to be made immediately if the blood sample cannot be dispatched rapidly. Hair roots tolerate handling by regular mail, but the test must be done within 5 days after receipt of the sample. These tests are not considered as a practical routine procedure in most laboratories. Thus the FMRP test is mainly used for large scale testing^{44,45} and for the assessment of difficult cases (i.e. patients with suggestive clinical phenotype but no expansion) but it is of limited use in most diagnostic laboratories. The test has been reported in the study of some fetuses but it has not been fully validated on chorion villi or amniotic fluid cells due to limited availability of material^{46,47,48,49}.

GENOTYPE

Detecting full expansions and/or methylation status

Using measurement of the CGG repeat, it may be difficult to distinguish a large premutation from a small full mutation. In such cases, determination of the methylation status is of great importance: premutations are not methylated while full mutations are usually methylated. However, fully expanded repeats that are completely or partially unmethylated have been reported in patients with a moderate or normal phenotype ("high-functioning" fragile X males)^{50,51}.

Southern blot analysis

The most commonly used enzymes are EcoRI and HindIII, which give normal fragments of approximately 5.2kb. Several equivalent probes are in use (see Figure 2).

Expanded repeats are unstable upon transmission from parent to offspring and can, therefore, show up as a diffuse smear rather than as a single enlarged band or a mixture of the two. If an expansion is suspected but the smear is too blurred to be sure about, digesting with BglII (which gives a normal fragment size of approximately 12 kb) will compress the smear enough to make it easily detectable. A female with a faint smear may be used as a control. As the amount of DNA can vary, the intensity of bands has to be compared in order to know if the normal bands are strong enough to exclude a faint smear.

A methylated full mutation (appearing as one or more sharp fragments) is usually stable somatically^{52,53} but is never transmitted stable within the family. If this appears to be the case, incomplete digestion or a restriction site polymorphism which prevents the enzymatic digestion should be checked for⁵⁴.

Tarleton *et al*⁵⁵ reported a point mutation within the CGG repeat which creates a new *EagI* restriction enzyme site and this appears as a deletion event.

When the methylation status is to be determined, EcoRI is combined with a methylation-sensitive enzyme such as *EagI* or *NruI*, which gives a fragment of approximately 2.8 kb from the normal, unmethylated FMR1 gene. Methylated normal alleles are not cut by methylation sensitive enzymes, and give rise to the normal 5.2-kb EcoRI fragment. Methylated and unmethylated expansions are indicated by the presence of bands and/or smears above 5.2 kb and 2.8 kb respectively. In females, an X-inactivation bias between the methylated and unmethylated normal fragments (skewed X-inactivation) is an indication that an expansion may be present, but an inactivation bias is not always associated with an expansion.

PCR analysis

PCR tests specifically optimized to detect large expansion and/or methylation status have been described. One of them is a PCR test combined with Southern blot followed by chemiluminescent probe detection which resolves both normal, premutation and full mutation alleles³⁹. Some tests were specifically modified in order to detect large expansions and/or methylation status: they involve a methylation-sensitive restriction enzyme³⁰ or bisulphite treatment of DNA prior to amplification^{40,41}. Weinhaeusel *et al.*⁴² have developed a methylation-sensitive polymerase chain reaction (MS-PCR) strategy that combines repeat-length and methylation analysis of the CGG-repeat and the promoters of the FMR1 and XIST genes. The allelic methylation of the latter opposes that of the FMR promoter and serves as an internal control and standard for semiquantitative analyses. Nevertheless, the interpretation of this test may be tricky in a female with a full mutation due to the presence of the methylated inactive normal X chromosome. A false-positive MS-PCR based diagnosis of fragile X syndrome has recently been reported to the assessors of the fragile X quality assessment scheme.

The methyl-sensitive PCR techniques are not suitable for early prenatal diagnosis since the tests are based on DNA methylation which is not completed in chorionic villi samples and prenatal testing with DNA from amniocentesis has not been fully evaluated.

Detecting premutations

Southern blot analysis

In the methylation sensitive digest, premutations are detected as fragments of 2.9 to about 3.3 kb in males and on the active X chromosome in females, whereas the 5.3 to 5.7 kb fragments correspond to the premutation on the inactive X chromosome. Note that the FMR1 promoter is fully methylated on normal inactive X chromosomes in

females. X-inactivation methylation of premutations should not be misinterpreted as an indication of the presence of a methylated full mutation.

It should be noted that in some female carriers of a premutation, biased X inactivation (skewing) can result in an atypical banding pattern (2.8 kb + expansion and 5.2 kb or 2.8 kb and 5.2 kb + expansion). Great care should be taken to ensure that the 2.8 and 5.2 kb bands are clearly aligned with the neighbouring tracks to exclude this possibility. A control probe, StA22, associated with StB12.3, has been developed which allows a check on the homogeneity of migration throughout the gel⁵⁶. This probe is very useful in cases of a small premutation and/or a premutation with skewed inactivation, and for the detection of migration artefacts (e.g. with an inappropriate DNA extraction protocol, with samples extracted from blood in Lithium Heparin tubes, sample overloading,).

Although most premutations can be detected in these conditions, it is often necessary to determine the size of small expansions more accurately. An alternative Southern blot test is possible with PstI digest (with StB12.3XX, Pfax3 or OX 0.55 probes⁵⁷) but the required increase in resolution could be more accurately achieved with CGG PCR.

PCR analysis

PCR analysis is very useful for accurate sizing under 120 CGGs, i.e. to discriminate between normal, grey zone and small premutation alleles. Only in the latter case, an estimation of the transition risk upon female transmission can be done¹⁸.

PRENATAL DIAGNOSIS

For prenatal diagnosis, PCR and Southern blot approaches have the same sensibility, sensitivity, and pitfalls as were discussed above for lymphocyte DNA analysis, with the exception that the use of a methylation sensitive method is not suitable on chorionic villi DNA because methylation of the full mutation is not always present at the 8th-10th week of pregnancy⁵⁸. Also, in contrast to lymphocytes and amniocytes, the FMR1 gene is not methylated on the inactive X chromosome in the chorionic villi of female fetuses.

A methylation sensitive analysis would lead to the division of a mutated signal into two classes, methylated and unmethylated fragments, which would be more difficult or impossible to detect. For this reason it is preferable to use only a methylation insensitive enzyme for prenatal Southern blots.

In prenatal diagnosis, the size of the expansion is the most important consideration. In the case of a signal in the large premutation/small full mutation range, determination of the methylation status normally is critical for distinction between the two classes of mutations and an *EagI*+*EcoRI* digest should be done on a DNA sample from amniotic fluid cells or fetal blood to give a definitive result.

Since DNA amounts and concentrations available from prenatal samples may be low, it can be difficult to exclude a very heterogeneous mutation that appears as a very faint smear. It is important to load similar amounts of DNA in all lanes if possible, and it may be useful to include another probe specific for another locus to demonstrate the presence of sufficient foetal DNA. In a female foetus, a normal signal is always present. It can be useful to compare the intensity of this signal to the expected intensity in order to be as sure as possible that a weak signal is not missed.

Note that indirect diagnosis with microsatellite markers is still of some value because of its speed in a prenatal diagnosis context, especially when Southern blotting is not possible or had inconclusive results, or when not enough DNA is available. Then a result may be rapidly obtained with a normal (non-risk) haplotype. But a determination of the expansion size is necessary when the at-risk haplotype is detected in the foetus. Of course, samples from appropriate family members are essential to determine the phase of the haplotype.

Furthermore microsatellites are the recommended way to detect/exclude maternal contamination of the fetal DNA sample which can lead to a false diagnosis, especially with the CGG PCR technique. We have detected significant maternal contamination in 3 out of 206 prenatal diagnoses. Some microsatellites markers are given in Table 5.

INTERPRETATION

The following size ranges of CGG repeats and the corresponding interpretations were suggested at the Best Practice meeting in Strasbourg.

Number of repeats	Result
<50 repeats	Normal range
50-58 repeats	Intermediate Alleles = grey zone
>58 – 200 repeats (not methylated on the active X)	Premutation
>200 repeats (usually abnormally methylated on the active X)	Full mutation.

Table 2 : Potential inaccuracy in sizing should be born in mind when interpreting the results based on this table. It is reasonable to accept a maximum error of +/- 4 % of the total repeat size.

Normal range

- Although minor changes in repeat numbers upon parental transmission have been described for alleles of 46-49 repeats, these are judged to be of no significance for genetic counselling.

Intermediate alleles = grey zone alleles

- Possible instability upon transmission, but transition to a full mutation allele in one generation has never been reported
- Because these alleles might display size instability upon transmission to offspring , referral to the local genetic counselling service for further family studies is recommended to clarify this.
- Prenatal diagnosis should be offered to women with 55 repeats and above.

The Strasbourg Meeting did not consider it necessary to perform further analysis (e.g. of AGG interspersions), as there is not enough research data available to enable quantitative predictions about the stability of such alleles. However AGG interruptions are generally thought to stabilize CGG arrays in this size range.

Premutation

- Unstable upon transmission. The risk for a female of having an affected child is proportional to the premutation size ¹⁸.
- About 20% of females carrying a premutation have premature ovarian failure (before 40y of age). This is important in genetic counselling of young females.

- Although a suspicion of FXTAS is an indication to search for a premutation, the prevalence of this late onset syndrome is not yet known and one should be cautious in specifying a risk for a relative found with a premutation in the context of carrier testing.
- The interpretation of a newly detected premutation allele depends entirely upon the reason for referral.
 - ◆ In "query carrier" referrals in known fragile X families, a premutation in a female denotes that she is a carrier, with a risk of having affected children. The risk for a female premutation carrier of having affected children is proportional to the size of the premutation (but potential inaccuracy in sizing should be kept in mind).
 - ◆ In an asymptomatic male from a fragile X family, a premutation denotes a "normal transmitting male"; the premutation will be transmitted, with little or no change in size, to all his daughters, but will not expand to a full mutation in one generation. In a diagnostic query (patient with mental retardation) interpretation of a premutation is more difficult. There is the possibility of mosaicism, with full mutations being present in tissues other than blood, as premutations are not usually associated with mental retardation. Nevertheless, this finding has implications for the family, as other members may be at risk of transmitting fragile X syndrome. It is recommended that at risk individuals are referred to the local Genetic Counselling service for further investigation.
- Confirmation of a prenatal result by amniocentesis could be offered in cases where a premutation has been found in chorionic villi DNA, in order to detect or exclude somatic mosaicism. No discrepancy had been observed by the meeting group, but these cases are rare.

Full mutation

- Males with full mutations will develop fragile X syndrome. Exceptions are extremely rare, and are likely to involve tissue or methylation mosaicism ^{50,51,59,60,61}
- Approximately 60% of females with full mutations are mentally retarded although their level of retardation is, on average, less severe than that seen in males. Many have some form of learning difficulty and behavioural problems.

REPORTING

Although local policy can vary with regard to reporting, some cases and suggested items to be included in the report are set out in Table 3.

Reason for referral	Report	
	Genotype	Interpretation
		Diagnostic
Southern	MALE AND FEMALE • This sample had no expansion / normal allele(s)	<ul style="list-style-type: none"> • Report the presence of normal allele(s) • Report that the fragile X diagnosis is unlikely but not ruled out (possibility of point mutation or gene deletion) • Report that she/he is not a carrier of the fragile X syndrome caused by an expansion
Southern +	MALE AND FEMALE • This sample had an	<ul style="list-style-type: none"> • Report the presence of an allele in intermediate range

PCR	allele in the intermediate range	<ul style="list-style-type: none"> Report that the fragile X diagnosis is unlikely but not ruled out (possibility of point mutation or gene deletion) MALE AND FEMALE : mention the indication of genetic counselling for the family 	<ul style="list-style-type: none"> MALE AND FEMALE : he/she is at risk of being a carrier of fragile X syndrome FEMALE : information about her risk of having affected children and about the possibility of prenatal diagnosis MALE AND FEMALE : mention the indication of genetic counselling for the family
Southern (and PCR)	MALE AND FEMALE <ul style="list-style-type: none"> This sample had a premutation 	<ul style="list-style-type: none"> Report the presence of a premutation 	
		<ul style="list-style-type: none"> A premutation is usually not associated with mental retardation. Report that the fragile X diagnosis is unlikely but not ruled out (possibility of tissue mosaicism or point mutation or gene deletion) he/she is a carrier of fragile X syndrome FEMALE : information about her risk of having affected children and about the possibility of prenatal diagnosis (and about POF) MALE AND FEMALE : mention the indication of genetic counselling for the family 	<ul style="list-style-type: none"> he/she is a carrier of fragile X syndrome FEMALE : information about her risk of having affected children and about the possibility of prenatal diagnosis (and about POF) MALE AND FEMALE : mention the indication of genetic counselling for the family If appropriate, e.g. in cases of presenile tremor/ataxia and/or dementia, give information on FXTAS.
Southern	MALE AND FEMALE <ul style="list-style-type: none"> This sample had a full mutation 	<ul style="list-style-type: none"> Report the presence of the full mutation 	
		<ul style="list-style-type: none"> Report that the fragile X diagnosis is confirmed FEMALE : she is a carrier of fragile X syndrome FEMALE : information about her risk of having affected children and about the possibility of prenatal diagnosis MALE AND FEMALE : mention the indication of genetic counselling for the family 	<p>FEMALE</p> <ul style="list-style-type: none"> she is a carrier of fragile X syndrome information about her risk of having affected children and about the possibility of prenatal diagnosis mention the indication of genetic counselling for the family
(PCR pre-screening)	<p>MALE</p> <ul style="list-style-type: none"> This sample had a normal allele This sample had no normal allele <p>FEMALE</p> <ul style="list-style-type: none"> This sample had one normal allele This normal allele is homozygous or hemizygous <p>FEMALE</p> <ul style="list-style-type: none"> This sample had two 	<ul style="list-style-type: none"> Report the presence of a normal allele Report that the diagnosis of fragile X syndrome is unlikely but not ruled out (possibility of point mutation or gene deletion or CGG expansion mosaicism) <p><i>No or preliminary report : Southern blot required</i></p> <ul style="list-style-type: none"> Report the possibility of homozygosity for this normal allele or the presence of an expansion based on the sensibility of the test used Note that complementary analysis has to be done to distinguish between normal homozygous female or a female with an expansion. Report the presence of two normal alleles Report that the fragile X diagnosis is unlikely but not ruled out (possibility of point mutation or gene deletion or CGG expansion mosaicism) 	<p><u>Preliminary reports can be issued but confirmation by a method which is able to detect large premutations and full mutations also is required.</u></p>

	normal alleles		

Table 3 : Items used as criteria for marking in the four fragile X External Quality Assessment schemes provided by EMQN since 2001.

“Best Practice guidelines for “reporting” are available at the EMQN web site (www.emqn.org). The following points should be included in each report:

- Date of referral / arrival noted
- Laboratory reference
- No typographical errors
- Individual reports issued
- Reason for referral restated : clear distinction between diagnostic testing, carrier testing and prenatal diagnosis
- Clear and concise report : “background” information about testing methodology, etc, be kept in the background
- Testing method and approximate repeat numbers mentioned or error limits provided
- Clear and concise conclusion, presented prominently
- Report dated and signed by two suitably qualified persons

TECHNICAL PROTOCOLS

Size Markers/Control samples

It is essential to include size markers in all types of Fragile X analysis. The most useful markers are those which lie at the borders between normal and intermediate allele and between premutation and between intermediate allele and premutation.

A cell line has been established for one female with a premutation allele of 59 repeats established by sequencing and is available from the American Type Culture Collection. Other cell lines from the Coriell Cell repositories (Camden, NJ) have been sequenced⁶².

For Southern blots it is useful to include a female with a small premutation on Southern - if the premutation is not distinguishable from the normal band, the gel has not run far enough. A useful marker will help to identify a partial digest which may well be mistaken for a mutated fragment⁵⁷. An alternative is to include a partial digest at least once (fragments resulting from an incomplete EcoRI digest have around 6.5; 9 and 10 kb and a 4.1 kb fragment appears also in EagI incomplete digestion with StB12.3).

CGG PCR

The primer sequences are taken from the references indicated. Individuals should consult the appropriate reference for full instructions and information on primer use.

Reference	Primer sequence	Position locus Genbank Accession L29074
Fu et al ³	5'-GCTCAGCTCCGTTTCGGTTTCACTTCCGGT-3' 5'-AGCCCCGCACTTCCACCACCAGCTCCTCCA-3'	13712-13723 13992-13963
	(CGG)n	13833-13892
Wang et al ²⁹	5'-TGACGGAGGCGCCGCTGCCAGGGGGCGTGC-3' 5'-GAGAGGTGGGCTGCGGGCGCTCGAGGCCCA-3'	13796-13825 13923-13897

Table 4 : Some primer references and positions. Note that there is a hot-spot of deletion of the CGG with the 5' breakpoint between position 13748 and 13780²⁶

Microsatellites Markers

A table of useful markers is set out below. FRAXAC1 and FRAXAC2 show essentially zero recombination with the (CGG)_n repeat. The high degree of linkage disequilibrium between FRAXAC1 and FRAXAC2 makes them less informative for indirect genotyping. DXS548, DXS297, and DXS8091 are widely used. Although less informative, DXS998 and DXS1215 are closest to the CGG repeat and may be of some interest. Although very rare, gene conversion, recombination events and reduction of full mutation to premutated or even normal allele have been described, and have to be considered in indirect diagnosis^{32,63,64,65,66,67,68}. The possibility of microsatellite instability or of a null allele (described for DXS548 by Vaisanen et al⁶⁶ and found in several chromosomes in our lab, unpublished results) should be taken into consideration.

Locus	Het	Fragment size	
DXS297 (VK23)	0.66	179-195 bp	5'-TTGGACTTCCCAAGCCTCCACAA-3' 5'-TTCTGAGTCTGTGCAGTGATTTGTCAG-3'
DXS998	0.57	113-119 bp	5'-CAGCAATTTTTCAAAGGC-3' 5'-AGATCATTATATAACCTCAAAGA-3'
DXS548 (RS46)	0.64	190-206 bp	5'-AGAGCTTCACTATGCAATGGAATC-3' 5'-GTACATTAGAGTCACCTGTGGTGC-3'
FRAXAC1	0.67	145-165 bp	5'-GATCTAATCAACATCTATAGACTTTATT-3' 5'-GATGAGAGTCACTTGAAGCTGG-3'
(CGG) _n			
FRAXAC2	0.80	145-165 bp	5'-GACTGCTCCGGAAGTTGAATCCTCA-3' 5'-CTAGGTGACAGAGTGAGATCCTGTC-3'
DXS1215	0.55	242-250 bp	5'-GGGCAAACATTAACCTCTC-3' 5'-GCCCTCTAAGTCATTACGCT-3'
DXS8091	0.76	89-111 bp	5'-CACATTCAGGTTCCACAGG-3' 5'-CAAGATCCAGGCAAAGTC-3'

Table 5 : Microsatellites

INTERNET RESOURCES

Department Clinical Genetics, Erasmus University Rotterdam, Pays-Bas (FMRP Test)

<http://www.eur.nl/FGG/CH1/fragx/>

FRAXA Research Foundation, USA

<http://www.fraxa.org/>

GeneClinics, University of Washington, Seattle, USA

<http://www.geneclinics.org>

The National Fragile X Foundation, San Francisco, USA

<http://www.nfxf.org>

Human Genome Epidemiology Network, USA

<http://www.cdc.gov/genetics/hugenet/reviews/FragileX.htm>

National Center for Biotechnology Information, USA

<http://www.ncbi.nlm.nih.gov/disease/FMR1.html>

National Library of Medicine, USA

<http://www.nlm.nih.gov/medlineplus/ency/article:001668.htm>

University of Western Ontario, London, Canada.

<http://www.icondata.com/health/pedbase/files/FRAGILEX.HTM>

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Donohoe Elizabeth	Galway	Ireland
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Acknowledgements : Didier DEVYS, Mireille COSSEE and Céline MOUTOU for helpful discussion.

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