



Association for Clinical Genetic Science
Part of the British Society for Genetic Medicine

Practice Guidelines for Molecular Diagnosis of Fragile X Syndrome

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1. NOMENCLATURE and GENE IDs

OMIM	Condition	Gene name	Gene map locus
309550	Fragile X Syndrome	<i>FMR1</i>	Xq27.3
309548	FRAXE	<i>FMR2</i>	Xq28

2. DESCRIPTION OF DISEASE

2.1 Fragile X Syndrome

Fragile X Syndrome is thought to be the commonest single-gene cause of learning disability features in humans with an estimated prevalence of 1 in 4000- 1 in 6000 males, where it causes moderate to severe intellectual and social impairment together with syndromic features including large ears and head, long face and macroorchidism¹. A fragile site (*FRAXA*) is expressible at the gene locus at Xq27.3, typically in 2-40 % of blood cells in affected males. The pathogenic mutation in most cases is a large expansion ('full mutation') in a CGG repeat tract in the first untranslated exon of the gene *FMR1*, which normally encodes the RNA-binding protein FMRP. Full mutations (from approximately 200 repeats upwards) result in hypermethylation of the DNA in and around the CGG tract, curtailed gene expression and no FMRP being produced²⁻⁴. Smaller expansions of the CGG repeat, or 'premutations' are not hypermethylated and hence do not cause Fragile X syndrome, but may show expansion into full mutations over one or more generations. Expansion from a premutation to a full mutation is invariably on transmission through female meiosis; paternal transmissions can be unstable but never result in a full mutation. Males with a full mutation, where tested, only have a premutation in their sperm. One case of male transmission of a full mutation has been reported⁵, but this has been disputed⁶. Females with a full mutation have a variable phenotype ranging from apparently normal (about 50%), to moderate mental and social impairment. Variable expressivity in females is possibly due to differences in the proportions of active and inactive normal and mutated X chromosomes in the relevant tissues. A small minority of

Fragile X cases are due to point mutations or deletions in the coding sequence rather than CGG repeat expansions^{7,8}; these do not exhibit fragile site expression or hypermethylation.

The distinct condition of FRAXE is caused by mutations in a second gene, *FMR2*, located slightly distal to *FMR1* in Xq28^{9,10} and is associated with a fragile site (*FRAXE*) which may be indistinguishable from *FRAXA* by conventional cytogenetics. In a fashion analogous to *FRAXA*, full *FRAXE* mutations are large expansions of a GCC repeat tract in the 5' UTR of *FMR2*, deriving from expansions of smaller premutation alleles; however the *FRAXE* disease phenotype is considerably less severe than the Fragile X Syndrome of *FRAXA* and lacks the specific syndromic features. The prevalence of *FRAXE* full mutations¹¹ is much lower than that of *FRAXA* (1 in 23 000) and no disease phenotype has yet been attributed to *FRAXE* premutations.

2.2 *FMR1* premutation-related disorders

Premutation alleles (variously described in publications as 55-200 or 59-200 repeats) were originally thought to have no clinical effect, but are now known to cause two quite different disease phenotypes at lower penetrance: primary ovarian insufficiency (POI) in females^{12,13} and Fragile X-associated tremor/ataxia syndrome (FXTAS)¹⁴.

Fragile X-associated primary ovarian insufficiency (FXPOI)

This condition is also known as premature ovarian failure (POF), but the term POI better encapsulates the broad spectrum of clinical manifestations seen in patients. A practical definition of POI is the presentation of amenorrhea in women before the age of 40 for four or more months in association with FSH levels in the menopausal range¹⁵. A varying degree of ovarian function is seen in 50% of women diagnosed with POI. Approximately 5-10% of women diagnosed with POI are able to conceive a viable pregnancy¹⁶; menopause is at the end point of the clinical spectrum for POI. On average, women with a premutation enter menopause five years earlier than non-carriers¹⁷.

Approximately 20% of premutation carriers develop POI compared to 1% in the general population. The risk of developing FXPOI is partly dependent upon the size of the premutation allele: a non-linear relationship has been reported for age at menopause and premutation size^{18,19}. The risk appears to be greater for premutations in the 80-100 CGG repeat range and less for premutations greater than 100 CGG repeats, although the risk cannot be excluded for any premutation size and the upper and lower size limits of POI risk are yet to be defined.

Fragile X-associated tremor/ataxia syndrome (FXTAS)

FXTAS is a late-onset neurodegenerative disorder found predominantly in male carriers of *FMR1* premutations. Increased transcription from the premutation allele and reduced FMRP results in accumulation of expanded CGG repeat mRNA which contributes to intranuclear inclusions and leads to the pathogenicity of FXTAS. Penetrance is age-related²⁰: it affects 17% of male premutation carriers aged 50-59, rising to 75% in patients aged 80. A number of studies based on small numbers suggest that females can also be affected with FXTAS; however the clinical symptoms are less severe in females and have some distinct differences from affected males²¹⁻²³. In contrast to POI, there is evidence of a linear correlation between phenotype and CGG repeat size: severity of symptoms is positively correlated²⁴ and age of onset negatively correlated with number of repeats, with most premutations (86%) found in FXTAS patients being >70 repeats²⁵.

Although FXTAS is primarily a premutation disorder, the situation regarding mosaic individuals with a full expansion mutation and a premutation is unclear²⁶. Recently FXTAS has been diagnosed in a mosaic individual with a full mutation and a premutation allele of 70 CGG repeats⁽²⁶⁾, in an individual with an unmethylated full-size expansion²⁷ and in an individual mosaic for unmethylated and methylated full-size expansion mutations ('methylation mosaic')²⁸. These findings may indicate that residual RNA expression from unmethylated full-size alleles with or without expression from unmethylated premutation alleles can lead to an overall

increase in *FMR1* mRNA giving rise to neurotoxicity and hence FXTAS in mosaic individuals. Premutation/full mutation mosaicism is not uncommon: depending upon the technique used, a detection rate of 12- 41% has been reported^{29,30}. Increasing use of more sensitive long-PCR techniques in commercial kits is likely to detect with greater sensitivity premutation/full mutation mosaics as well as methylation mosaics in blood leucocytes, but this is not necessarily indicative of mosaicism in the brain tissue.

Other possible premutation phenotypes

There have been reports suggesting a high rate of autism spectrum disorder (ASD) and attention-deficit hyperactivity disorder (ADHD) symptoms in boys with the premutation who presented as probands³¹, while in male premutation carriers from Fragile X families a high rate of autism and developmental delay has been reported³². In women, premutations have also been linked with fibromyalgia, hypothyroidism and multiple sclerosis^{24, 33}. However, many of these studies are small or do not reflect unbiased populations, so larger prospective studies are needed to determine the full clinical phenotype of premutations.

Reduced levels of the Fragile X protein (FMRP) have been reported in some premutation carriers³⁴, the reduction correlating with increasing number of CGG repeats in the premutation range^{35, 36}. It is currently unclear what level of FMRP is critical for cognitive function; moreover, data are sparse on the effect of larger premutations (over 110 CGG repeats) on FMRP levels owing to their relative rarity. It should also be considered that for larger repeat sizes there is a greater chance of undetected mosaicism for a full mutation. Furthermore, *FMR1* CGG repeats are unstable in somatic tissues during early embryogenesis and significant tissue mosaicism has been reported in cheek cells³⁷ and in skin fibroblasts^{38, 39} compared with peripheral blood.

This presents a dilemma when a premutation is detected in a patient referred for Fragile X syndrome: is the premutation itself the cause of symptoms, is it a coincidental finding or could the patient be mosaic for a premutation and a full mutation. Given the general nature of the presenting phenotype in most probands and the likely frequency of size mosaicism between tissues, it would be unwise to ascribe causality of a premutation detected in blood to a Fragile X-like phenotype until alternative diagnoses and (if appropriate) alternative tissue analysis has been explored.

3. REFERRAL CATEGORIES FOR FRAGILE X TESTING

3.1 Diagnostic testing: Fragile X Syndrome

Common reasons for diagnostic referral will include developmental delay, learning/behavioural difficulties, speech delay, autistic features, ADD/ADHD, social dysfunction, poor eye contact and challenging behaviour as well as physical features such as large head, large ears, macroorchidism, hand flapping/biting and dysmorphic facies. Although the physical Fragile X phenotype is well-defined in post-pubertal males, this is not true of females and young children where the full mutation phenotype is variable and often subtle. This means that Fragile X diagnostic testing is typically carried out on a very broad range of patients; consequently the pickup rate is low (in most laboratories, only around 0.6% of males tested will be positive for a full mutation). While it is theoretically possible to increase the specificity of the test by clinical pre-selection of adult patients, this is more difficult for children in the age group under 10 years (which comprise the vast majority of diagnostic referrals, since early diagnosis of Fragile X syndrome is of crucial importance to inform other members of the family of their risk of having affected offspring).

In order to avoid the risk of missing a true Fragile X case, it has been common practice to test all patients for whom a specific request for Fragile X testing has been made; this can, however, lead to a high level of inappropriate *FMR1* testing in patients with clinical phenotypes inconsistent with Fragile X syndrome. In 2010 the UK Genetic Testing Network (UKGTN) approved testing criteria for Fragile X diagnosis in male and female patients as well as for

carrier testing (www.ukgtn.nhs.uk). **While the testing policy for Fragile X referrals must be agreed locally with referring clinicians, it should, as far as possible, comply with the UKGTN criteria.**

If Fragile X testing is not specifically requested and the clinical information lists any features which might be suggestive of Fragile X, DNA can be extracted and stored. The opportunity can then be given for the referring doctor to request a Fragile X test at a later date.

If array-CGH is available, it may be appropriate to prioritise this test over specific Fragile X testing as it is more likely to detect an abnormality of clinical significance in the majority of referrals for which Fragile X would be requested; however, any such policy should be balanced against the inevitable increase in reporting times as well as the strong possibility of finding abnormalities which do not necessarily account for the patient's phenotype.

In any case, laboratories should have a clear written policy on acceptance criteria for Fragile X testing and *FMR1* premutation-related disorders.

3.2 Diagnostic testing: POI and FXTAS

Referrals for POI/POF may be tested by conventional karyotype to rule out sex chromosome abnormalities before Fragile X testing is initiated, but if only a Lithium Heparin sample is received it would be preferable to request an additional sample in an EDTA tube for the Fragile X analysis rather than risk compromising the assay. Referrals for FXTAS may in some cases also request molecular tests for other neurological gene mutations, in which case the most appropriate and cost-effective testing strategy should be agreed between the laboratory and the referring clinician.

3.3 Carrier testing

Testing for carrier status in a known Fragile X family is normally carried out only with the approval of a Clinical Geneticist, as such a test may have predictive implications for the patient (POI/FXTAS) as well as for their reproductive options if a premutation is detected. 'Carrier' testing may also detect full mutations in women with no obvious symptoms of Fragile X. **Therefore any referrals without clinical symptoms received from non-Genetics specialists should be treated with caution and referred to the local Clinical Genetics centre. Testing of asymptomatic patients under 16 should not be carried out unless there is a specific recommendation to do so from a Clinical Geneticist.**

4. CLASSIFICATION OF *FMR1* ALLELES AND RISKS OF EXPANSION

4.1 CGG repeat expansion mutations

Historically *FMR1* alleles have been classified according to size and instability; such a classification is empirical and the boundaries are not hard and fast. The definitions of normal, intermediate and premutation alleles in size terms have led to much confusion with different size limits being set by different authors, so it is worth re-stating the empirical definition of these three categories.

Normal allele: up to 45 repeats. An allele that gives a normal phenotype and is inherited stably in the vast majority of meiotic transmissions. Alleles in this size range account for over 98% of those found in most populations studied, with 30 the modal number in Caucasians⁴⁰.

Intermediate allele: 46-58 repeats. Alleles in this size range pose perhaps the biggest single challenge to Fragile X molecular diagnosis in terms of interpretation, reporting and genetic counselling, as they represent the overlap zone between stable normal alleles and unstable premutations. In addition, it is not clear whether alleles in the intermediate range show clinical involvement in abnormal phenotypes such as POI, FXTAS or developmental delay. Evidence

for a clinical involvement of intermediate alleles is patchy and contradictory and should not preclude alternative diagnoses.

Intermediate alleles are often transmitted stably, but show a greater tendency to unstable transmission with increasing size in this range⁴¹. The magnitude of change is incremental and does not lead to expansion to a full mutation in a single generation. There is a strong correlation between the stability of an intermediate allele and the presence of interspersed AGG motifs within the CGG tract: most normal and intermediate alleles consist of (CGG)₉ or ₁₀AGG(CGG)₉AGG(CGG)_n, the distal tract of CGG accounting for most of the length variation between alleles. Instability is associated with a) total length of repeat; b) fewer interspersions and c) length of the longest uninterrupted CGG tract⁴¹⁻⁴³. Alleles with pure CGG repeat tracts or with only one AGG interspersion are considerably more unstable than alleles with at least two AGG interspersions. However the degree of instability is greater for larger alleles within the intermediate size range. While most unstable transmissions are confined to the high intermediate size range of 50-58 repeats and are in most cases small incremental changes⁴¹, there are at least three documented cases of alleles below 60 repeats having converted to a full mutation in a single generation: two of 59 pure repeats⁴⁴ and one of 56 pure repeats⁴⁵, the latter having expanded from a paternally-inherited 52-repeat allele with two AGG interspersions. Hence, the long-held suspicion that loss of AGG interspersions is a major determinant of instability has been demonstrated in practice.

It follows that the ability to analyse interspersion patterns would mark a 'pure' CGG intermediate allele at a greater risk of expansion, while presence of two or more AGG interspersions would imply that the allele is likely to be transmitted stably. Nolin *et al.*⁴³ studied 457 maternal transmissions of alleles in the size range 45-69 and found nine which had expanded to a full mutation in one generation: all were 59 repeats or more with no AGG interspersions. Notwithstanding the rarity of expansion to a full mutation from alleles under 59 repeats, interspersion analysis may be a useful aid to genetic counselling whenever an intermediate allele is detected, even if at the moment we cannot give precise risks of expansion. **As a precaution and to reflect standard errors in sizing between laboratories, prenatal diagnosis should be offered to all women with an allele of 55 CGG repeats or greater.**

Premutation allele: 59- 200 repeats (not hypermethylated). One which expands in the majority of transmissions, usually by more than 2 repeats and progressively more in each generation, and whose ultimate destiny is to become a full mutation. Premutation alleles can expand to a full mutation in a single generation, with a size-dependent probability (note that most premutations tested are found to lack AGG interspersions, though this observation is biased by ascertainment usually *via* a Fragile X proband).

Premutation alleles as mentioned above are associated with FXPOI and FXTAS but not with clinical symptoms of Fragile X syndrome in the majority of cases. However, large premutations (close to 200 repeats) are often mosaic with a full mutation, whether due to genuine size mosaicism or 'methylation mosaicism' (i.e. where the same-sized expansion is detected in both unmethylated and methylated forms- since hypermethylation of the CGG repeat defines a full mutation, regardless of size).

It should be appreciated that there is a genuine overlap between these allelic categories and no definition based on size will be entirely free of both Type I and II errors: there will be occasional examples of instability in the 'normal' range, while it is perfectly possible for alleles in the 'premutation' range to be transmitted stably (especially so if they have been ascertained independently of a full mutation, and hence free of ascertainment bias). Moreover there may be marked population and ethnic variation in allele stability, correlated with AGG interspersion variability and/or genetic background, which may account for the differences in preferred allele size categories across the world. Some guidelines specify 55 repeats as the lower limit of the 'premutation' class to take into account the observation of an expansion from 56 repeats to a

full mutation in a single generation⁶⁸, but in view of the rarity of such events **we recommend that 59 repeats is a more realistic lower boundary of a premutation for diagnostic reporting purposes, with the understanding that allele categories based on size alone are approximate and may be revised either by new empirical evidence, family history or AGG interspersion data.**

Various estimates for premutation carrier frequency have been reported for different populations, ranging from 1 in 113 in Israel⁴⁶ to 1 in 382 in women from USA⁴⁷. Tassone *et al.*⁴⁸ report from newborn screening a premutation frequency of 1 in 209 in females and 1 in 430 in males. (Note, however, that any estimate of premutation prevalence is highly sensitive to the definition used, as 55 repeats is a relatively common allele and hence any survey such as these which includes 55 repeats in the premutation range will inevitably find a much higher prevalence than those which include only 59 repeats or more). Approximately 75% of the premutation alleles detected in the USA population are below 70 CGG repeats. These studies, regardless of population differences, indicate that alleles of 55 repeats or more are not infrequent and thus it is not unusual to detect such alleles in patients referred for diagnostic Fragile X testing.

The implications of such a finding for the individual and family require careful counselling combined with an accurate assessment of risk of expansion to a full mutation, particularly on maternal transmission. In the past much of the evidence for risk of expansion to a full mutation was ascertained from families with molecular confirmation of Fragile X syndrome, but these data could not be reliably used for premutation carriers without a family history of Fragile X. Nolin *et al.*⁴⁹ found that the transmission stability of premutation alleles differed significantly for women with and without a family history of Fragile X, and the risk of expansion to a full mutation is greater in families with a known Fragile X proband- as expected, owing to the bias of ascertainment in the latter. **These data provide a useful basis for risk estimates for expansion which can be used for counselling both in known Fragile X families and those where a premutation may have been independently ascertained.** These estimates are particularly useful when no detailed analysis of the internal structure of the repeats has been carried out.

The presence or absence of AGG repeats can have dramatic effect on the risk of expansion to a full mutation⁵⁰. Published risks which take into account the CGG repeat structure are based on small sample size but illustrate the potential magnitude of change: for example, the risk of expansion to a full mutation for a premutation of 75 CGG repeats with no AGG interspersions is predicted to be 77% whereas the risk is only 12% for an allele of the same size but with two AGGs. For larger premutation alleles, interspersion data is of limited clinical utility since there will always be a significant risk of expansion. The decision to test and report AGG interspersions must therefore depend on local policy and the nature of the referral. Other genetic factors, such as the local flanking haplotype, may also be associated with instability; however there is a degree of autocorrelation and linkage disequilibrium between such haplotypes and the AGG interspersion alleles^{41,51-53}.

Full mutation: >200 repeats, hypermethylated. Methylated large expansions account for >99% of cases of clinical Fragile X syndrome. The full mutation almost always leads to hypermethylation of the DNA in and around the expanded repeat tract, even on the active X chromosome; there are, however, rare cases of 'high-functioning' or mildly-affected males with full-size expansions in the absence of significant hypermethylation^{54,55}. Females with a full expansion mutation may or may not have Fragile X symptoms or may be mildly affected; all, however, have a 50% risk of transmitting a full expansion mutation.

Summary of recommended allele classification:

Normal:	up to 45 repeats
Intermediate:	46 - 58 repeats
Premutation:	59 - approximately 200 repeats, unmethylated
Full mutation:	Greater than approximately 200 repeats, methylated

4.2. Coding sequence mutations

Deletions and point mutations in the *FMR1* coding sequence are thought to comprise a very small minority of Fragile X pathogenic mutations, though studies have been limited and mutation screening for FRAXA is not at present cost-effective except perhaps in older males with the clinical phenotype who test negative for the CGG repeat expansion. However, the advent of next-generation sequencing may be able to fill the small gap in sensitivity offered by current testing regimes.

5. MOLECULAR DIAGNOSIS OF FRAXA

Testing strategies

Fragile X mutations can be identified by various molecular techniques: the most commonly used are fluorescent PCR (fPCR) across the CGG repeats, Southern blot hybridization and specialist commercial long-PCR kits (such as the Amplidex™ system from Asuragen or the FragilEase™ kit from Perkin Elmer). **Conventional fPCR** across the CGG repeat is rapid, inexpensive, and can detect alleles up to around 120 repeats but will not detect larger expansions and is prone to preferential amplification of the smaller alleles in females. **Southern blotting** can detect all sizes of expansion and can also determine methylation status but is laborious, requires careful optimization and does not have the resolution to give a precise allele size; it also requires much more DNA than PCR-based methods. **Commercial long-PCR-based *FMR1* kits** have the unique advantages of being able to detect normal, premutation and full mutation alleles and to give them a precise size estimate; in addition, some are able to determine methylation status and AGG interspersion patterns. These may be considered expensive for primary exclusion testing, but may be a viable alternative to Southern blotting whenever a secondary test is required.

5.1 Fluorescent PCR

Conventional fPCR analysis is sufficient to detect all normal alleles and therefore to exclude a diagnosis of Fragile X syndrome in the vast majority of diagnostic referrals, subject to two main provisos: that mosaicism for a normal and a full mutation allele is absent or very rare, and that the PCR test will not detect rare point mutations and deletions within the *FMR1* coding sequence, nor any FRAXE mutations unless a separate PCR is carried out for the *FMR2* gene. It may also be noted that in the Finnish population a tandem duplication has been reported which may give rise to a false negative result if reporting on fPCR results alone⁵⁶.

Mosaicism for a normal allele and full expansion mutation is rare but has been reported^{57,58}, and the risk may be higher for individuals with a family history of Fragile X; a retrospective study of male patients across several laboratories found 1-2% of all Fragile X patients to be mosaics (EMQN best practice guidelines⁶⁸). **It is therefore recommended that all patients with atypical or low-strength PCR results, or with a confirmed family history of Fragile X, be tested by Southern blotting and/or specialist kit analysis and not solely by fPCR.**

Traditionally, laboratories have used fPCR as a pre-screen and proceeded to Southern blot analysis only on those samples which fail to amplify (males) or show a single allele (females). Various PCR primer sets and methods have been used, a selection of which is detailed in the Appendix; if desired, FRAXA and FRAXE can be duplexed in a single PCR⁵⁹. The PCR products may be visualized on an agarose gel with ethidium bromide staining and more precise sizing carried out using an automated sequencer and genotyping software. Some fluorescent

PCR assays may be less efficient than others at detecting alleles over 50 repeats especially in females due to preferential amplification of the smaller allele. This creates a potential 'gap' in sensitivity of the diagnostic test if the Southern blot method used does not resolve small expansions at boundary of intermediate/premutation size range.

5.2 Southern blot analysis

Southern blot analysis using probe StB12.3⁶⁰ or Ox1.9⁶¹ on DNA that been double-digested with a methylation-sensitive enzyme (such as *Bst*ZI, *Eag*I, *Nru*I or *Bss*HII) and a non-methylation-sensitive enzyme (such as *Eco*RI or *Hind*III) allows for the detection of premutation alleles and full mutation expansions. Methylated (inactive) normal alleles are cut only by one enzyme to give a 5.1 kb fragment, while unmethylated (active) normal alleles are cut by both enzymes to yield a 2.8 kb fragment. Male premutation carriers will manifest as single enlarged fragments above 2.8 kb, while in females four fragments corresponding to the normal active, mutant active (2.8 kb and above), normal inactive and mutant inactive (5.1 kb and above) will be observed if X-inactivation is random. In both males and females full expansions are seen as a band significantly larger than 5.1 kb. Full mutations can be distinguished from large premutations by their methylated status, even on the active X chromosome.

The double digest, while a good all-round technique for detecting most expansions, may have limits to its sensitivity in cases of full mutations which appear as diffuse smears due to somatic mosaicism (particularly in females, where the presence of the normal allele may draw attention away from any faint expanded fragments). Also, extreme skewing of X-inactivation in favour of the mutant allele may result in small premutations being hard to detect in females. Hence, care should be taken in the interpretation of blots where the signal strength is low or background is high. In cases where an expansion is suspected but the smear is too diffuse to be sure about, a single digest with *Bgl*II giving a normal fragment size of 12 kb will often compress the smear enough to make it easily detectable. Greater resolution and more accurate sizing of premutation alleles can be achieved by a single *Pst*I digest and using a probe close to the CGG repeats such as pfxa3/Ox0.55^{3,61}. This blot is, however, seldom required now because of the improved efficiency of PCR techniques in detecting and sizing premutation alleles.

5.3 Specialist commercial kits

Recently, commercial PCR-based *FMR1* kits (e.g. AmplidexTM from Asuragen; FragilEaseTM from PerkinElmer) have come to market and are being implemented in service. The AmplidexTM *FMR1* PCR kit, now in widespread use, supports two different PCRs: a gene-specific PCR which gives full-length product peaks for each allele size and a CGG repeat-primed PCR (a TP-PCR combined with the gene-specific PCR – subsequently referred to as TP-PCR). Both kits will amplify normal and expansion alleles; however only TP-PCR will give information on the AGG interruptions, which appear as 'troughs' or antimodes in the row of stutter peaks. The AmplidexTM *FMR1* kit therefore allows for the detection and sizing of normal and premutation alleles, detection of full mutations and a measurement of the number and position of AGG sequences. The basic kit does not, however, provide information on the methylation status. As full mutations are almost always methylated, the detection of a full-sized expansion mutation in a proband is consistent with a diagnosis of Fragile X. There is, however, a need to test for methylation status of large borderline pre/full mutation alleles in females referred for Fragile X carrier testing. The AmplidexTM *FMR1* mPCR kit allows for the detection of methylation status, extent of methylation and the sizes of premutation and normal alleles. In both kits the full expansion mutations are not specifically sized but are detected as expansions greater than 200 repeats. If methylation studies have not been undertaken, where relevant this should be stated on the report and information given regarding further testing.

It is not yet known whether mPCR would detect all cases of apparently skewed X-inactivation in females that are detected by Southern blot, though it may allow for a more quantitative estimate of methylation percentages; in any case, a skewed X-inactivation pattern observed in blood

may not be reflected in other tissues and hence is not a reliable predictor of the severity of the Fragile X phenotype.

Specialist kits may thus be used either as an alternative to Southern blot analysis or as a second-line test to reduce the frequency of Southern blotting; as well as being less labour-intensive they also have the advantage of much lower turnaround times. However, rare cases of atypical results obtained by PCR or mPCR may still require Southern blotting, and the use of kits in prenatal diagnosis should be subject to local validation (see 5.4 below).

5.4 Prenatal diagnosis

Fluorescent PCR analysis may initially be carried out on the CVS and both parents which, if informative, should show whether the normal maternal allele has or has not been inherited by the fetus. However, note that the risk of potential false-negative interpretations of PCR results due to maternal cell contamination of the CVS is particularly acute in Fragile X testing because of the wide discrepancy in size between normal and expanded alleles, with consequent preferential amplification of the former. Therefore **Southern blot or specialist kit analysis is advisable in order to obtain a conclusive result in prenatal diagnosis. If specialist kit analysis is to be the sole method used, it must first be validated locally on prenatal DNA samples.** Use of two or three separate CV fronds for PCR is common practice, but is dependent upon the receipt of sufficient sample for Southern analysis. Obstetricians should be requested to take **at least 15-20 mg of CVS** as long as the fetus is not compromised. **Good communication between laboratory and Clinical Genetics staff is important throughout the prenatal. Laboratories should resist pressure to issue an 'interim' or premature report based on fPCR analysis alone.** If a reportable result cannot be obtained from Southern blot analysis owing to technical difficulties, a result based either on specialist kit analysis or fPCR analysis supported by linkage analysis (see 5.5 below) may still be reported in some circumstances, such as when the fetus can clearly be shown to have inherited the mother's normal repeat allele and the low-risk haplotype.

When analyzing Southern blots for prenats it should be borne in mind that methylation is not usually established in chorionic villus sample (CVS) tissue and therefore the distinction between premutation and full mutation must be made on size alone (though sometimes somatic mosaicism can be seen in unmethylated full mutations). A double digest, while not necessary in a blot for prenatal diagnosis, could be useful to give a more precise sizing of a borderline premutation/full mutation allele. If space and sample quantity allows, it would be good practice to load both a single and a double digest of the CVS on the same gel, but if sample quantity is limiting then a single digest would give a better chance of a result as the signal would not be split. Note that CVS extractions often tend to contain more RNA than extractions from blood samples; hence DNA concentration readings can sometimes be overestimates and more DNA sample may therefore need to be loaded to compensate.

Although prenatal diagnosis is also possible using amniotic fluid (AF) or fetal blood samples, these are not ideal as they are taken at a much later stage in pregnancy than CVS; moreover, a typical AF sample would need culturing for at least 10-14 days to yield a sufficient DNA sample for Southern blot analysis.

If a large premutation-sized allele is detected in the CVS DNA, the possibility of size- or methylation-mosaicism for a full mutation cannot be ruled out, although none has been reported. In such cases, further prenatal investigations using DNA from amniotic fluid or fetal blood could be considered, but this must be balanced against the increased risk of miscarriage. In any case, failure to detect a full mutation in prenatal DNA cannot exclude this possibility in other tissues. **Therefore when counselling patients clinicians need to be aware that in very rare cases a definitive diagnosis/prediction may not be possible.** For female fetuses, there is an additional risk of Turner Syndrome: the incidence of mosaic Turner syndrome is reported to be up to 5% in female fetuses of mothers carrying the full mutation⁶². Karyotyping

or QF-PCR carried out in parallel with Fragile X testing on the prenatal sample should be routine.

5.5 Linkage analysis

The utility of microsatellite markers for linkage analysis has diminished with the advent of the direct tests, but it is nevertheless recommended that laboratories have access to linkage methods for occasional unusual or difficult cases, such as for prenatal diagnosis where Southern blotting has failed or not given a clear result. The markers DXS548, FRAXAC1 and FRAXAC2 can be combined in a convenient multiplex PCR⁶³; these are highly informative and show negligible recombination with the CGG repeat. For extra informativity, the FRAXE repeat can also be included (see 6 below).

6. MOLECULAR DIAGNOSIS OF FRAXE

Given the rarity of FRAXE full mutations relative to FRAXA and the lack of a clearly defined clinical phenotype, it is not thought necessary to test for FRAXE in all referrals for 'Fragile X' testing, although it may be an integral part of the PCR exclusion protocol in some centres. Several centres are able to offer FRAXE testing when required for relatives in known families, or to confirm a diagnosis of FRAXE where the PCR test has failed to exclude it. A Southern blot using a double digest may be employed on similar lines to that used for FRAXA, typically using *HindIII* and *NotI* as the enzyme combination in conjunction with probe OxE20⁹. However, beware of a rare *HindIII* polymorphism generating a fragment of ~6 kb which may be mistaken for a full mutation⁶⁴; hence any large discrete expansions of around this size should be checked for the polymorphism before reporting. If PCR analysis is the primary test, up to 30 repeats is considered normal, but the extent of the intermediate/premutation range is uncertain owing to the relative rarity of such alleles; hence, any allele over 30 repeats should be regarded as potentially unstable. It is less likely that prenatal diagnosis would be offered to FRAXE carriers than to FRAXA carriers, but laboratories should continue to follow local clinical guidance regarding the extent of FRAXE molecular testing in their region.

7. REFERENCE MATERIALS (CONTROLS)

In the past there has been a considerable variation in the size estimates reported for a particular *FMR1* allele by different centres, particularly for intermediate and premutation alleles where the spread of 'stutter' artifacts is broader. Although such discrepancies have become less evident with the adoption of new fluorescent sequencer technology, it is important that the accuracy of a patient's result is not compromised by local variation in sizing technique (this is true whether or not sizes are quoted on reports, as it may affect the categorization of alleles as recommended above). It is therefore desirable that any controls run on PCR tests should have their number of repeats verified against a nationally agreed standard reference control.

Standard reference materials are now available from a number of sources. In the UK, a panel of five prototype reference materials has been developed and approved as the first WHO international reference standard (code: 08/158) by the National Institute of Biological Standards and Control (www.nibsc.ac.uk) to include heterozygous normal, premutation and full mutation females, premutation and full mutation males. In the USA, DNA containing triplet repeat numbers ranging from 20 to 118 has been prepared and verified by the National Institute of Standards and Technology in Gaithersburg, USA, and is marketed as a single kit (SRM 2399). Meanwhile, useful DNA's from cell lines with CGG repeats which have been sized by sequencing are available from NIGMS Human Cell Repository at Coriell Cell Repositories⁶⁶ (see 10.4 below).

8. REPORTING GUIDELINES

It is not necessary to report the estimated size in repeats of most normal-sized alleles, which can safely be reported as 'within the normal size range', provided that this range is quoted. Equally, many full mutations will be impossible to size with any great precision by Southern blot alone especially if there is any somatic mosaicism. However **for alleles in the intermediate or premutation size range, or at the borderline of the normal/intermediate ranges, it is imperative to give as accurate an estimate as possible of the allele size, together with a quote of the allele size definitions (normal, intermediate and premutation). All sizes quoted for intermediate, premutation and full mutation alleles should be qualified as 'approximately' or a confidence limit stated.** Specialist *FMR1* kits and in some cases optimised fPCR would allow for reasonably accurate sizing, with confidence limits, of premutation and intermediate alleles; these sizes can be used to give the risk of expansion to a full mutation for females if appropriate, depending upon the family history (see 4.1 above).

The wording of diagnostic reports will obviously vary to some degree depending upon the precise circumstances of the referral and the result obtained, and also whether an estimate of allele size is quoted; the following phraseology is recommended for some common scenarios.

Full mutation found in a symptomatic patient:

'Southern blot analysis of the *FMR1* gene detected a full expansion mutation (*quote approximate size, if this can be determined- and state if mosaicism for premutation detected*) in (Patient). **This result supports/confirms a diagnosis of Fragile X Syndrome.** We recommend that the patient be referred to (local Consultant Clinical Geneticist) who can provide further advice, explain this finding to the family and take further samples as appropriate.'

(Or using specialist *FMR1* kit, without methylation test):

'Analysis of the *FMR1* gene detected a full expansion mutation in (Patient). Although we have not tested methylation status, it is very likely that the expansion is methylated as it is in the full mutation size range (>200 repeats). **This result supports/confirms a diagnosis of Fragile X Syndrome.** We recommend that the patient be referred to (local Consultant Clinical Geneticist) who can provide further advice, explain this finding to the family and take further samples as appropriate.'

Normal PCR allele found in symptomatic male/two normal alleles in symptomatic female:

'**We found no evidence of an *FMR1* expansion mutation in (Patient).** PCR analysis detected an allele/two alleles in the normal size range (up to 45 repeats *-or quote approximate size*). **This result does not support a diagnosis of Fragile X.** Please note that this test will not detect very rare cases of point mutations or deletions in the *FMR1* gene.'

One normal allele found in symptomatic female, no expansion found by Southern blot/*FMR1* kit:

'**We found no evidence of an *FMR1* expansion mutation in (Patient).** PCR analysis detected one allele in the normal size range (up to 45 repeats). Analysis by Southern blot/ (long-PCR™) kit also detected a normal allele with no evidence of an expanded allele. These results indicate that the patient is homozygous for an *FMR1* CGG repeat allele in the normal range (i.e. has two copies of a normal-sized allele). **This result does not support a diagnosis of Fragile X.** Please note that this test will not detect very rare cases of point mutations or deletions in the *FMR1* gene.'

*Full mutation allele found in (unaffected) female relative of Fragile X patient:

'Analysis of the *FMR1* gene by Southern blot/ (long-PCR™) kit detected a full expansion mutation in (Patient).

This result shows that (Patient) has a full expansion mutation.

The patient has a high risk of having children affected with Fragile X Syndrome. We recommend that (patient) be offered prenatal diagnosis in any future pregnancy.' (*if indicated by age of patient*)

*Premutation allele found in female relative of Fragile X patient:

Premutation range: 59 – 200 repeats

'A small DNA expansion (premutation) of approximate size (n) (+/- CL if known) repeats was detected in the *FMR1* gene of (Patient). **Therefore (Patient) is a carrier of an *FMR1* premutation.**

Premutations are likely to show instability and further expansion in future generations, with a risk of expansion to a full mutation. Prenatal diagnosis can be offered (*if indicated by age*) to the patient in any future pregnancy.

Female premutation carriers are at increased risk of developing Fragile X-associated primary ovarian insufficiency (FXPOI). Other family members may be at risk of inheriting the mutation and can now be offered testing if and when appropriate. We recommend referral to a Clinical Genetics Service.' (*if not already referred*)

*Premutation allele found in male relative of Fragile X patient:

Premutation range: 59 – 200 repeats

'A small unmethylated DNA expansion (premutation) of approximate size (n) (+/- CL if known) repeats was detected in the *FMR1* gene of (patient). **Therefore (Patient) is a carrier of an *FMR1* premutation.**

The result has clinical implications for (Patient) who is at risk of developing Fragile X-associated tremor and ataxia syndrome (FXTAS). The average penetrance of FXTAS in male premutation carriers of 50 years and above is 39% and increases to 75% for male premutation carriers of 80 years and above (Jacquemont *et al.* 2004, JAMA 291: 460-469).

The result also has implications for other family members who may have inherited this mutation, especially any daughters who will be obligate carriers with a risk of having children with Fragile X syndrome. Carrier screening of those relatives at risk can now be offered if and when appropriate. We recommend referral of this family to a Clinical Genetics service.' (*if not already referred*)

*It is assumed that these types of report will always be addressed to a Clinical Genetics specialist; if this is not the case then appropriate recommendation to refer the patient and family for Clinical Genetics counselling should always be added.

Premutation allele found in symptomatic male/female, no family history of Fragile X:

Premutation range: 59 – 200 repeats

'A small DNA expansion (premutation) of approximate size (n) (+/- CL if known) was detected in the *FMR1* gene of (Patient). Premutation alleles are not usually associated with Fragile X syndrome, although the possibility that larger premutations may lead to cognitive impairment or

autism in some patients cannot be excluded¹. There is also a small possibility of mosaicism for a full mutation in other tissues, but we found no evidence of mosaicism for a full mutation in the sample tested. (*if Southern blot or specialist kit testing undertaken*)

This result does not support a diagnosis of Fragile X syndrome and is unlikely to explain the clinical symptoms seen in this patient. However, the premutation is likely to be unstable and prone to further expansion in the next generation. Therefore the result has implications for other family members who may be at risk of inheriting the mutation.

A copy of this report has been sent to (Local Consultant Clinical Geneticist). Please contact the Clinical Genetics Service (*contact details*) to discuss the case and to arrange for genetic counselling, follow-up and further genetic testing in the family if appropriate.

1. Hagerman, R. *et al.* 2010. *Molecular Autism* **1**: 12. (www.molecularautism.com/content/1/1/12)

Intermediate allele found in male/female, no family history of Fragile X:

'PCR analysis of the *FMR1* gene detected an expanded allele of approximately ___ repeats in (patient). This allele lies in the intermediate size range (46-58 repeats) which is not thought to be associated with developmental delay/learning disability, but which might display size instability in future generations.

This result does not support a diagnosis of Fragile X syndrome.

#EITHER: We recommend that (patient) be referred to the local Clinical Genetics service for genetic counselling and further family studies, which may help to determine the stability of this allele in the family.

#OR: If you would like to discuss this result, please contact the local Clinical Genetics Service who will provide advice and can arrange for further tests within the family as appropriate.'

#depending upon local practice as agreed between the laboratory and Clinical Genetics service.

The salient message on all reports should be clear and concise, highlighted in **bold** and easily understandable by all clinicians. **In particular the term 'carrier' is ambiguous and should not be used when referring to a full mutation female.** Where abbreviations are used in a report, a key should be used for clarity e.g. I = Intermediate, etc. **Allele sizes, where quoted, from the intermediate range upwards and especially in the full mutation range, should be described as approximate.** All reports should comply with the ACGS Best Practice Guidelines on Reporting (www.acgs.uk.com) and also follow guidance from the relevant NEQAS schemes.

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10. APPENDIX

10.1 PCR primers

a) CGG repeat in *FMR1* (FRAXA)

FMR1-c: gct cag ctc cgt ttc ggt ttc act tcc ggt
 FMR1-f: agc ccc gca ctt cca cca cca gct cct cca

Reference: Fu *et al.*⁶⁷

FXD tga cgg agg cgc cgc tgc cag ggg gcg tgc
 FXE gag agg tgg gct gcg ggc gct cga ggc cca

Reference: Wang *et al.*⁵⁹

b) GCC repeat in *FMR2* (FRAXE)

598: gcg agg aag cgg cgg cag tgg cac tgg g
 603: cct gtg agt gtg taa gtg tgt gat gct gcc g

Reference: Knight *et al.*⁹

c) Linkage analysis/haplotyping

DXS548 A: aga gct tca cta tgc aat gga atc

DXS548 B: gta cat tag agt cac ctg tgg tgc
FRAXAC1 A: gat cta atc aac atc tat aga ctt tat t
FRAXAC1 B: aga ttg ccc act gca ctc caa gcc t
FRAXAC2 A: gac tgc tcc gga agt tga atc ctc a
FRAXAC2 B: cta ggt gac aga gtg aga tcc tgt c

Reference: Chiurazzi *et al.*⁶³

10.2 Southern blot probes

StB12.3 References: Oberlé *et al.*²; Rousseau *et al.*⁶⁰
Contact: Prof. Jean-Louis Mandel (jlmandel@igbmc.fr)
Ox1.9 Reference: Nakahori *et al.*⁶¹
Ox0.55 = pfxa3 References: Nakahori *et al.*⁶¹; Yu *et al.*³
OxE20 (FRAXE) Reference: Knight *et al.*⁹

10.3 Custom-made *FMR1* kits and protocols

Amplidex from Asuragen: www.nomoresouthernblots.com (includes useful Webinar series)

FragilEase from Perkin Elmer: www.perkinelmer.com

Fragile X analyte-specific reagents (ASR) from Abbott: www.abbottmolecular.com

10.4 Reference materials:

NIST, USA: www.nist.gov/srm

CRMGEN: www.crmgen.org

NIBSC: www.nibsc.ac.uk

Coriell Cell Repositories: <http://ccr.coriell.org/nigms/>