

Practice Guidelines for molecular diagnosis of Fragile X Syndrome.

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1. NOMENCLATURE and GENE IDs

Table 1.

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

Fragile X Syndrome is probably the commonest single-gene cause of learning disability in humans with an estimated prevalence of 1 in 4000 to 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 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', do not cause Fragile X syndrome but may show expansion into full mutations over one or more generations. Females with a full mutation have variable phenotypes ranging from apparently normal (about 50%), to moderate mental and social impairment, with or without fragile site expression. Variable phenotype 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^{5,6}; these do not exhibit fragile site expression or hypermethylation.

Premutations are now known to cause two quite different disease phenotypes at lower penetrance: premature ovarian failure (POF) in females^{7,8} and fragile X-associated tremor/ataxia syndrome (FXTAS) in males⁹; these have a different pathogenic basis to Fragile X Syndrome, probably due either to subtle reductions in FMRP levels or to RNA gain-of-function effects.

The distinct condition of FRAXE is caused by mutations in a second gene, *FMR2*, located slightly distal to *FMR1* in

Xq28^{10,11} 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 mutations is much lower than that of *FRAXA*, and no disease phenotype has yet been attributed to *FRAXE* premutations.

3. REFERRAL CATEGORIES FOR FRAGILE X TESTING

Common reasons for diagnostic referral will include developmental delay, learning/behavioural difficulties, speech delay, autistic features, Asperger syndrome, 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 usually well established in post-pubertal males, this is not true of females and young children where the full mutation phenotype is variable and often subtle. As early diagnosis of Fragile X syndrome is of crucial importance to inform other members of the family of their risk of having affected offspring, this means that most Fragile X diagnostic tests will be carried out on a very broad range of patients with a consequently inevitable low pickup rate (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 patients, this would be difficult for children in the age group under 10 years which comprise the vast majority of diagnostic referrals. ***It is therefore good practice to test all patients for whom a specific request for Fragile X testing has been made.*** If Fragile X testing is not specifically requested at this stage and the clinical information lists any features which might be suggestive of Fragile X, DNA can be extracted and stored and the opportunity given for the referring doctor to request a Fragile X test at a later date. Depending upon local practices, some centres may prefer to test any appropriate referral with or without a specific request. In any case, laboratories should have a clear written policy on acceptance criteria for Fragile X testing.

Unless there is a confirmed family history of Fragile X, samples are likely to be referred simultaneously for cytogenetic karyotyping and hence molecular Fragile X analysis may be carried out from the same (lithium heparin) blood sample; however, some laboratories have experienced problems of high failure rates or aberrant DNA migration with samples extracted from blood in lithium heparin tubes (particularly from frozen blood). In such cases it is obviously preferable to request a sample in an EDTA tube if Fragile X testing is required, rather than risk compromising the assay.

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 (POF/FXTAS) as well as for their reproductive options; hence any referrals received by other routes without clinical symptoms should be treated with caution and referred to the local Clinical Genetics centre. **Testing of asymptomatic patients under 16 year of age should not be carried out unless there is a specific recommendation to do so from a Clinical Geneticist.**

4. MOLECULAR DIAGNOSIS OF FRAXA

4.1 Normal alleles

The CGG repeat in *FMR1* exhibits a stable polymorphism in the general population from 6 to around 50 repeats (see section 4.5 below); alleles in this size range account for over 98% of those found in most populations studied. PCR analysis is sufficient to detect all such normal alleles¹² and therefore to exclude a diagnosis of Fragile X Syndrome in the vast majority of diagnostic referrals, subject to two 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. While a CMGS study did not find significant evidence of true mosaicism for a normal and full mutation allele, one should nevertheless beware of artifactual PCR bands which may occur in the normal range being misinterpreted as normal alleles.

It is now standard procedure for laboratories to use PCR as a pre-screen and only proceed to Southern blot analysis 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 PCR¹³; it has been reported that this has the benefit of suppressing artifactual PCR bands seen when FRAXA is run alone. Visualization and sizing of the PCR products may be carried out either by radioactive labelling and autoradiography, fluorescent labelling with automated sequencer and analysis software or silver staining. For simple exclusion testing in males, agarose gel/ethidium bromide staining may be employed, but this has insufficient resolution for precise sizing or for exclusion testing in females. **It is essential to include appropriate size markers and size controls;** in particular for agarose/ethidium visualization, a control sample of approximately 50 repeats should always be included.

4.2 Premutations

Premutations are small to medium-sized expansions ranging from approximately 55-200 repeats which are *unmethylated*

on active X-chromosomes, but are subject to methylation due to X-inactivation in females. While the smaller premutations may be detected by PCR analysis (depending upon the assay conditions), larger premutations can only reliably be detected by Southern blot analysis. As the distinction between a premutation and a full mutation (see 4.3 below) is more likely due to methylation status rather than the exact size of the repeat, it is common to utilize a double enzyme digest with one methylation-sensitive enzyme such as *BstZI*, *EagI*, *NruI* or *BssHII*, combined with another enzyme such as *EcoRI* or *HindIII* to give a convenient size of fragment for resolution of the expansions and their methylation status.

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.

Using a probe such as StB12.3¹⁴ or Ox1.9¹⁵ this approach is effective for the detection of premutation alleles of all sizes, which in males 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. As mentioned above, large premutations may be distinguished from small full mutations by their unmethylated status on active X chromosomes, assuming that this pattern is replicated in the tissues where *FMR1* is expressed and hence that FMRP activity is not significantly curtailed. Smaller premutations may need to be sized more precisely by PCR analysis to distinguish them from intermediate alleles (see section 4.5 below).

For premutation carriers, family follow-up and prenatal diagnosis should be strongly recommended. The probability of conversion to a full mutation on maternal transmission in a single generation is low for premutations of 59-70 repeats, but rises to >90% for premutations of more than 90 repeats¹². Allowing for possible errors in sizing of +/-2 repeats, we recommend that female carriers of alleles **57 repeats or more should be offered prenatal diagnosis.**

N.B. **It appears that some fluorescent PCR assays may be less efficient than radioactive PCR 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. Laboratories should therefore ensure that their PCR technique is optimized to reliably detect expanded alleles at sizes which can be readily resolved by Southern blotting (usually 60 repeats or more). There is also some variation in the ability of PCR assays to resolve two alleles differing by a single repeat in females; unless these cases can be confidently scored as heterozygous, Southern blot analysis should also be carried out.** It is recommended that all patients with a high prior risk or with a confirmed family history of fragile X be tested by Southern blotting as well as by PCR

4.3 Full mutations

Full mutations are large expansions creating CGG tracts ranging in size from around 200 repeats to a thousand repeats or more, which can only reliably be detected by Southern blot analysis. Full mutations are also characterized by **methylation of the DNA in and around the expanded repeat tract, even on**

the active X chromosome. Using a double digest as described above, methylated expansions are indicated by the presence of one or more bands or smears above 5.1 kb (in contrast to premutations, several different-sized expansions may be observed due to somatic mosaicism). Approximately 20% of full mutation patients also show some mosaicism for a premutation, which may be the same size ('methylation mosaics') or more usually, distinctly smaller than the full mutation.

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*III giving a normal fragment size of 12 kb will often compress the smear enough to make it easily detectable. For detecting small premutations, a single *Pst*I digest using a probe close to the repeat array such as pfxa3/Ox0.55^{3,15} will give the best resolution as the normal fragment size is 1.0 kb, but is also likely to exacerbate the problem of diffuse smears in mosaic full mutations.

4.4. Prenatal diagnosis

When analysing 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 tend to contain more RNA than extractions from blood samples; hence O.D. readings can be misleading and more DNA sample should therefore be loaded to compensate.

Additional PCR analysis may 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 foetus. 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 analysis should be the method of choice to obtain a conclusive result in prenatal diagnosis.*** 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 foetus 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 PCR analysis alone. If a reportable result cannot be obtained from the Southern blot analysis owing to technical difficulties, a result based on PCR analysis of the CGG repeat ***supported by the use of linkage analysis*** (see 4.7 below) may still be reported in some circumstances, such as when the foetus can clearly be shown to have inherited the mother's normal repeat allele and the low-risk haplotype.

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

4.5. Intermediate alleles

Alleles in the size range of approximately 46 to 58 repeats 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. These 'intermediate' alleles are often transmitted stably, but are more likely to show unstable transmission with increasing size in this range¹⁶. Most unstable transmissions of intermediate alleles are small increments of only one or two repeats; frequently, an intermediate allele may show both stable and unstable transmissions within the same family. In addition, it is not known whether any alleles in the intermediate range show clinical involvement in abnormal phenotypes such as POF, FXTAS or developmental delay.

The stability of an intermediate allele appears to correlate with the presence of two or more interspersed AGG motifs within the CGG tract; most normal and intermediate alleles consist of (CGG)₉ or (CGG)₁₀AGG(CGG)₉AGG(CGG)_n, the distal tract of CGG accounting for most of the length variation between alleles. Since unstable premutations are usually either pure CGG or possess only the proximal AGG interspersion, it is assumed that the loss of one or both AGG motifs or their conversion to CGG in an intermediate allele would predispose it to instability. It follows that sequencing of intermediate alleles or other methods of determining interspersion pattern (such as *Mnl*I digestion) could, in theory, predict their stability^{17,18}. In practice, the determinants of stability are probably more complex¹⁹ and not enough longitudinal data are available at present on the stability of alleles of different sizes and interspersion patterns for this to be a reliable predictor; hence interspersion analysis is not currently used in routine diagnostic Fragile X testing.

Where an intermediate allele is detected in a new diagnostic referral, it will most likely be in the absence of any family history or previous *FMRI* testing so assessment of risk must be based upon published empirical data. Recent data have shown (a) that most unstable transmissions are confined to the high intermediate size range of 50-58 repeats²⁰; and (b) that the smallest documented premutation to have converted to a full mutation in a single generation is 59 repeats²¹. Meanwhile, there is no consistent or strong evidence for a clinical involvement of intermediate alleles. Given these considerations it is appropriate to categorize alleles ***under 50 repeats as normal*** (likely to be stable), ***50-58 repeats as intermediate*** (may show instability) and ***59 repeats or more as premutations*** (likely to show instability and with a possibility of conversion to a full mutation in one generation).

For carriers of intermediate alleles, *limited family follow-up may be recommended to determine the stability of the allele within the family*, which may be the most useful guide to risk given the likely effect of genetic background on allele stability.

Summary of recommended allele classification:

Normal: <50 repeats

Intermediate: 50-58 repeats

Premutation: 59-approximately 200 repeats, unmethylated

Full mutation: Greater than approximately 200 repeats, methylated

4.6 Reference Materials

In the past there has been a considerable variation in the size estimates reported for a particular *FMRI* 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 in future, controls run on PCR tests should have their number of repeats verified against a nationally agreed standard reference control.

Standard reference materials will shortly be available from a number of sources. 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). In the UK, a panel of five prototype reference materials has been developed with heterozygous normal, premutation and full mutation females, premutation and full mutation males by the CRMGEN consortium (www.crmgen.org). These materials are now being developed as WHO standards by the National Institute of Biological Standards and Controls, with a target approval date of late 2006. 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 Repositories²². For a summary of contact information, see section 9.3.

4.7 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 PCR²³; these are highly informative and show negligible recombination with the CGG repeat (see 9.1c below). For extra informativity, the FRAXE repeat can also be included (see 5 below).

5. 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 OxE20¹⁰. However, beware of a rare *HindIII* polymorphism generating a fragment of ~6 kb which may be mistaken for a full mutation²⁴. 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 unlikely that prenatal diagnosis would be offered to FRAXE premutation carriers, but laboratories should continue to follow local clinical guidance regarding the extent of FRAXE molecular testing in their region.

6. PREMUTATION PHENOTYPES

6.1 Premature Ovarian Failure (POF)

POF, defined as cessation of menstruation before the age of 40, occurs with a penetrance of approximately 20% in carriers of an *FMRI* premutation⁷. In addition, age of menopause is on average five years earlier in premutation carriers compared to non-carriers. This creates an extra genetic counselling implication for female carriers of a premutation regardless of whether or not the ascertainment is via a Fragile X proband. It is therefore recommended to report this implication whenever a premutation is detected in a diagnostic referral (see Reporting Guidelines below).

It is less clear whether testing for an *FMRI* premutation should be implemented routinely for all women referred solely for POF, given an expected pick-up rate of 4% overall and 12% in patients with familial POF⁸. Although the direct clinical benefit of the test to the patient is low, there would be a significant benefit to her relatives found to be carriers who would be at risk not only of POF but of having offspring with Fragile X syndrome. However it may be necessary to impose strict referral criteria until further research on the effects of premutations on ovarian function and the stability of premutations ascertained in this way has been undertaken. In the meantime, the present situation of most laboratories testing for premutations upon request seems appropriate.

6.2 Fragile X tremor and ataxia syndrome (FXTAS)

The phenotype of FXTAS is less well-defined and incorporates many of the features of other, commoner aging diseases. It is reported to have a penetrance of 17% in male premutation carriers over 50 rising to 75% in patients aged 80 or over²⁵; there is as yet no conclusive evidence for such an effect in females. Again, the present policy of testing for premutations only upon request (mainly from neurologists) is appropriate: although there is some evidence of an increased frequency of premutations in referrals for SCA testing²⁶, this would warrant further research before implementing as an extra routine diagnostic test. The risk of FXTAS in later life further complicates the issue of carrier testing in males: when deciding whether to test asymptomatic males in known Fragile X families, the implications for the patient of a predictive test

for FXTAS should be balanced against the need to establish which side of the family carries the mutation.

7. REPORTING GUIDELINES

The wording of diagnostic reports will obviously vary to some degree depending upon the precise circumstances of the referral and the result obtained, but the following phraseology is recommended for some common scenarios.

Fragile X full mutation found in a symptomatic patient:

'Southern blot analysis of the *FMR1* gene detected an expanded allele of approximately ___kb above normal size (*or: in the full mutation range*) 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.'

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

'We could find no evidence of a Fragile X (FRAXA) expansion mutation in this patient. PCR analysis detected an allele/two alleles in the normal size range (under 50 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:

'We could find no evidence of a Fragile X (FRAXA) expansion mutation in this patient. No DNA expansion was detected in the *FMR1* gene using Southern blot analysis. PCR analysis detected a single normal allele (under 50 repeats); we assume (patient) to be homozygous for the normal 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:*

'Southern blot analysis of the *FMR1* gene detects an expanded allele of approximately ___kb above normal size/___repeats (*or: in the full mutation range*) in (patient). **This result shows that (patient) has a full Fragile X mutation.** 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:*

'Southern blot/PCR analysis of the *FMR1* gene detects an expanded allele of approximately ___kb above normal size/___repeats (*or: in the premutation size range*) in (patient). **This result shows that (patient) carries a Fragile X premutation.** We recommend that (patient) be offered prenatal diagnosis in any future pregnancy (*if indicated by age of patient*).

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

'Southern blot/PCR analysis of the *FMR1* gene detects an expanded allele of approximately ___kb above normal size/___repeats (*or: in the premutation size range*) in (patient).

This result shows that (patient) carries a Fragile X premutation. The result has implications for relatives of (patient), particularly his daughters who will be obligate carriers of the premutation.

*The above three report templates are based upon the assumption 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:

'Southern blot/PCR analysis of the *FMR1* gene detects an expanded allele of approximately ___kb above normal size/___repeats (*or: in the premutation size range*) in (patient).

This result shows that (patient) carries a Fragile X premutation.

Premutations are not thought to be associated with developmental delay/learning disability, therefore this allele is likely to be coincidental to the patient's phenotype. However, the premutation may be unstable during meiosis and prone to further expansion leading to a risk of Fragile X syndrome in descendants. In addition, premutations have been found to be associated in some cases with premature ovarian failure (*females*). We recommend that (patient) be referred to the local Clinical Genetics Service for genetic counselling and investigation of relatives who may be at risk of inheriting the premutation.'

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 (50-58 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. 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.

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 should be described as approximate. Please refer to the CMGS Best Practice Guidelines on Reporting (<http://www.cmgs.org/>) for further guidance.

8. REFERENCES

1. Chudley, A.E. and Hagerman, R.J. 1987. Fragile X Syndrome. *J. Pediatr.* **110**, 821.
2. Oberlé, I. *et al.* 1991. Instability of a 550-base pair DNA segment and abnormal methylation in Fragile X Syndrome. *Science* **252**, 1097.
3. Yu, S. *et al.* 1991. Fragile X genotype characterized by an unstable region of DNA. *Science* **252**, 1179.
4. Verkerk, A. *et al.* 1991. Identification of a gene (*FMR-1*) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in Fragile X Syndrome. *Cell* **65**, 905.
5. Hirst, M. *et al.* 1995. Two new cases of FMR1 deletion associated with mental impairment. *Am. J. Hum. Genet.* **56**, 67.
6. De Boule, K. *et al.* 1993. A point mutation in the *FMR-1* gene associated with fragile X mental retardation. *Nature Genetics* **3**, 31.
7. Allingham-Hawkins, D.J. *et al.* 1999. Fragile X Premutation is a significant risk factor for premature ovarian failure: The International Collaborative POF in Fragile X Study- Preliminary Data. *Am. J. Med. Genet.* **83**, 322.
8. Murray, A. 2000. Premature ovarian failure and the *FMRI* gene. *Sem. Reprod. Med.* **18**, 59.
9. Jacquemont, S. *et al.* 2003. Fragile X premutation tremor/ataxia syndrome: molecular, clinical and neuroimaging correlates. *Am. J. Hum. Genet.* **72**, 869.
10. Knight, S.J.L. *et al.* 1993. Trinucleotide repeat amplification and hypermethylation of a CpG island in *FRAXE* mental retardation. *Cell* **74**, 127.
11. Gu, Y. *et al.* 1996. Identification of *FMR2*, a novel gene associated with the *FRAXE* CCG repeat and CpG island. *Nature Genet.* **13**, 109.
12. Fu, Y-H. *et al.* 1991. Variation of the CGG repeat at the Fragile X Site results in genetic instability: resolution of the Sherman paradox. *Cell* **67**, 1047.
13. Wang, Q. *et al.* 1995. A rapid, non-radioactive screening test for fragile X mutations at the FRAXA and FRAXE loci. *J. Med. Genet.* **32**, 170.
14. Rousseau, F. *et al.* 1991. Direct diagnosis by DNA analysis of the Fragile X syndrome of mental retardation. *New Eng. J. Med.* **325**, 1673.
15. Nakahori, Y. *et al.* 1991. Molecular heterogeneity of the fragile X syndrome. *Nucl. Acids Res.* **19**, 4355.
16. Murray, A. *et al.* 1997. The role of size, sequence and haplotype in the stability of *FRAXA* and *FRAXE* alleles during transmission. *Hum. Mol. Genet.* **6**, 173.
17. Eichler, E.E. *et al.* 1994. Length of uninterrupted CGG repeats determines instability in the *FMRI* gene. *Nature Genet.* **8**, 88.
18. Kunst, C.B. and Warren, S.T. 1994. Cryptic and polar variation of the Fragile X repeat could result in predisposing normal alleles. *Cell* **77**, 853.
19. Gunter, C. *et al.* 1998. Re-examination of factors associated with expansion of CGG repeats using a single nucleotide polymorphism in *FMRI*. *Hum. Mol. Genet.* **7**, 1935.
20. Macpherson, J.N. *et al.* 2004. FRAXA intermediate and premutation alleles among novel diagnostic referrals: significance and reporting procedure. Presentation at CMGS Spring Meeting, Newcastle.
21. Nolin, S.L. *et al.* 2003. Expansion of the Fragile X CGG repeat in females with premutation or intermediate alleles. *Am. J. Hum. Genet.* **72**, 454.
22. O'Connell, C.D. *et al.* 2002. Standardization of PCR amplification for fragile X trinucleotide repeat measurements. *Clin. Genet.* **61**, 13.
23. Chiurazzi, P. *et al.* 1999. DNA Panel for interlaboratory standardization of haplotype studies on the Fragile X syndrome and proposal for a new allele nomenclature. *Am. J. Med. Genet.* **83**, 347.
24. Blayau, M. *et al.* 1998. FRAXE: the *HindIII/OXE20* restriction polymorphism is not a rare variant. *Hum. Genet.* **103**, 626.
25. Jacquemont, S. *et al.* 2004. Penetrance of the fragile X-associated tremor/ataxia syndrome (FXTAS) in a premutation carrier population: initial results from a California family-based study. *JAMA* **291**, 460.
26. Macpherson, J.N. *et al.* 2003. Observation of an excess of fragile-X premutations in a population of males referred with spinocerebellar ataxia. *Hum. Genet.* **112**, 619.

9. APPENDIX

9.1 PCR Analysis

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.* 1991

FXD: tga cgg agg cgc cgc tgc cag ggg gcg tgc

FXE: gag agg tgg gct gcg ggc gct cga ggc cca

Reference: 13

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: 10

c) Linkage analysis/haplotyping

DXS548 A: aga gct tca cta tgc aat gga atc

B: gta cat tag agt cac ctg tgg tgc

FRAXAC1 A: gat cta atc aac atc tat aga ctt tat t

B: aga ttg ccc act gca ctc caa gcc t

FRAXAC2 A: gac tgc tcc gga agt tga atc ctc a

B: cta ggt gac aga gtg aga tcc tgt c

Reference: 23

Recommended PCR conditions for linkage multiplex (per reaction):

Reagent	Amount
10 x PCR buffer	1.5 µl
25 mM MgCl ₂	0.9 µl
2 mM dNTP mix	1.5 µl
Glycerol	0.75 µl
FRAXAC1A*	4.5 pmol
FRAXAC1B	4.5 pmol
FRAXAC2A*	1 pmol
FRAXAC2B	1 pmol
DXS548A*	2 pmol
DXS548B	2 pmol
<i>Taq</i> polymerase (5 unit ml ⁻¹)	0.15 µl
dH ₂ O	make up to 14 µl

* Label primers with different fluorescent dyes for easy resolution.

Thermal Cycling Conditions:

Temp (°C)	Time (minutes:seconds)	Cycles
95	1:30	} 30
65	1:00	
72	1:30	
72	7:00	1

9.2 Southern Analysis

Probes:

Probe	References	Contacts
StB12.3	2,14	Dr Valérie Biancalana bianca@faust.u-strasbg.fr Prof. Jean-Louis Mandel mandel@igbmc.u-strasbg.fr
Ox1.9	15	
Ox0.55 (pfxa3)	3, 15	
OxE20 (FRAXE)	10	

9.3 Reference materials

Source	Contacts
NIST, USA	Barbara C. Levin PhD barbara.levin@nist.gov www.nist.gov/srm
CRMGEN	http://www.crmgen.org/
Coriell Cell Repositories	http://ccr.coriell.org/nigms/