



Best Practice Guidelines for Molecular Analysis of Friedreich Ataxia

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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. 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.

Nomenclature and gene ID

See table 1. The preferred abbreviation is FRDA as the use of FA may lead to confusion with Fanconi anaemia. Also X25 is not widely used as the gene name.

Description of the disease

Friedreich ataxia is the most common hereditary ataxia with an estimated prevalence of 1 in 50,000. It is an autosomal recessive neurodegenerative disorder characterised by progressive ataxia of all 4 limbs, ataxia of gait, loss of deep tendon reflexes, loss of position and vibration sense and pyramidal weakness of the legs. Motor nerve conduction velocities remain normal, but sensory action potentials are affected. Cardiomyopathy and diabetes mellitus are also seen with variable penetrance. The molecular pathogenesis of FRDA is reviewed by Puccio and Koenig (2000).

The most common DNA abnormality seen in FRDA is the homozygous expansion of a polymorphic GAA repeat in intron 1 of the frataxin (X25) gene (9q13), found in 98% of FRDA chromosomes. The carrier rate for this expansion has been estimated at 1 in 90 individuals in French and German populations (M. Koenig), but this figure is undoubtedly different in other populations. Normal alleles contain between 8 and 33 GAA repeats, with pathological alleles containing 90 to 1300 repeats. Repeat numbers between 34 and 100 have been termed premutations

and can expand into a full mutation in further generations. This is complicated by the presence of interruptions to the pure GAA repeat on some chromosomes which may have a stabilising effect. Pathogenic range expansions result in a significant reduction in the expression of frataxin and the resultant physiological manifestations. There is an inverse correlation between the size of the expansion and the age of onset of the symptoms, indicating that smaller, pathogenic range expansions allow a greater level of expression of frataxin than larger expansions. Normal onset of the symptoms is before the age of 20. Approximately 15 point mutations have been reported to date in affected individuals heterozygous for a pathogenic range GAA expansion (Cossée et al, 1999). In general the clinical manifestations of compound heterozygotes are similar to individuals with homozygous expansions. An exception to this is the common G130V mutation which tends to lead to an atypical FRDA with a milder form of the disorder (Bidichandani et al 1997). 1 deletion has been found which results in an apparent homozygous expansion in current molecular tests.

Common reasons for referral

Patients are most commonly referred from neurology clinics because of early onset ataxia. Due to the difficulty in distinguishing classical FRDA from some of the juvenile spinal cerebellar ataxias, requests for both FRDA and SCA testing are common. Although loosely applied in FRDA referrals, Harding diagnostic criteria (see 3.1, below) can be used to differentiate between typical and non-typical FRDA cases. Significant numbers of patients not displaying all of the Harding diagnostic criteria (non-typical FRDA) can be homozygous for the GAA expansion (McCabe et al 2000). Vitamin E deficiency can mimic the symptoms of FRDA and should be considered, as Vitamin E deficiency is a controllable condition.



Harding Diagnostic Criteria 3.1

In 1981, A. E. Harding studied 115 patients with Friedreich's ataxia (FA) and proposed what are commonly known as 'Harding's' criteria for the clinical diagnosis of this autosomal recessive syndrome. The mandatory clinical diagnostic criteria which emerged from this paper include: (a) an age of onset < 25 and definitely < 27 years, (b) ataxia of all four limbs, (c) ataxia of gait, (d) lower limb areflexia and (e) presumed or proven autosomal recessive inheritance. The frequency of other findings such as dysarthria, extensor plantar responses, abnormal lower limb vibration sensation and joint position sensation varied depending on the duration of the disease. Although considered important "secondary diagnostic criteria" for entry into Harding's study, dysarthria was noted in only 60% of patients five years after disease onset, and extensor plantar responses in only 88.7% of patients overall.

Approaches and protocols

A range of techniques is available for molecular diagnostic testing in FRDA. These guidelines are not intended to be prescriptive, but are intended to present a general strategy and to highlight advantages and pitfalls for some of the techniques in use in various centres. Most laboratories only carry out GAA repeat length analysis.

General strategy.

1. Appropriate patients selected for molecular analysis (referrals for FRDA / SCA testing)
2. Screen for GAA repeat expansion in affected individual
 - a. PCR amplification of frataxin intron 1 GAA repeat
 - b. Southern blot analysis of GAA repeat region
 - c. Triplet repeat PCR
3. Mutation detection on genomic DNA in GAA expansion heterozygotes
4. Characterisation of pathogenic mutation
5. Devise test to detect mutation
6. Carrier / pre-symptomatic testing in at-risk relatives
7. Linkage analysis

PCR analysis

PCR sizing of the GAA repeat using the intron 1 primers as described by Filla et al (1996) and a long PCR protocol can routinely be used to identify homozygous normal, homozygous expanded and heterozygous individuals. There is however general recognition that the expanded allele may fail to amplify in a small percentage of heterozygous individuals. Primer sets

generating larger amplicons from the normal allele are claimed to alleviate this selective amplification of the normal (smaller) allele Campuzano et al (1996). Triplet repeat PCR (TP-PCR) Warner et al (1996) based on GAA7 primers will identify expansions that may be problematic using standard PCR (Schmitt et al). This method will reliably detect expanded alleles in both homozygous and heterozygous expanded individuals. The detection of the normal (non expanded) allele in heterozygotes, while possible, is quite difficult using TP-PCR. The results of TP-PCR will also be affected by the presence of interruptions in the GAA repeat. TP-PCR may be most useful as a pre screen for expansions prior to standard long range PCR or southern blotting.

Southern Blotting

On Southern blots an exon 1 probe can be used to detect the intron 1 GAA repeat region (Durr et al 1996). The probe detects non expanded fragments of 2.4kb, 5.6kb and 8.2kb when genomic DNA is cut with BsiHKA1, EcoRV and EcoR1 respectively. While the BsiHKA1 enzyme allows superior resolution and a more accurate estimation of GAA repeat numbers, larger expansions can appear as a smear and may be difficult to detect. Using a 1.3% agarose gel to resolve the BsiHKA1 digested DNA can reduce the smearing and thus improve the detection of the expanded allele (M Koenig, personal communication). BsiHKA1 would thus appear to be the enzyme of choice. Several groups have reported the appearance of unexpected bands when using EcoR1 digested genomic DNA, possibly attributable to "star" activity. It may be advisable to replace EcoR1 with Sst1 when fragment sizes in this range are desired.

Mutation Detection

FRDA patients heterozygous for a GAA expansion should be considered for mutation screening. No confirmed FRDA patient to date has presented with homozygous point mutations and no GAA repeat expansion. All are homozygous for a GAA expansion or a compound heterozygote for a GAA expansion and an inactivating frataxin mutation. As each laboratory tends to have its preferred mutation screening techniques a discussion on mutation detection strategies is not appropriate and is beyond the scope of this text. The G130V mutation has been seen in several European populations and in the USA and may be useful in a mutation pre-screen, especially in the case of an atypical presentation as described by Bidichandani et al 1997. The exon primer sets described by Campuzano et al (1996) have been reliably used to amplify the frataxin exons for mutation screening protocols. The human gene mutation database (Cardiff) should be notified of all new frataxin mutations.



Linkage analysis

There is some evidence of locus heterogeneity in Friedreich Ataxia (Kostrzewa et al, 1997), with families demonstrating typical FRDA symptoms not linked to the FRDA 9q13 locus. Linked markers may be used to exclude FRDA in expansion negative "affected" sibs, if they do not share the same genotype.

Useful markers surrounding the FRDA locus that can be used in linkage analysis are D9S202 (FR1), D9S886 (FR2), D9S887 (FR7), D9S888 (FR8), D9S889 (FR5), (Rodius et al 1994), D9S1845, D9S1859, and D9S1862 (Montermini et al. 1995).

Clinical interpretation

Reporting results of GAA repeat sizing and point mutation analysis

Initial results indicated that GAA repeat numbers above 200 were pathogenic. This has since been modified to repeat numbers above 90. Repeat numbers between 34 and 100 have been termed pre-mutations with the possibility of both unchanged transmission or transmission as an expanded allele.

- *Detection of 2 pathogenic range GAA expansions* - should be reported as diagnostic of FRDA. Carrier status / pre-symptomatic testing can be offered to relatives of patient. There is a very low possibility of a deletion in the frataxin region giving rise to an apparent homozygous result. Testing of parental samples, if available, should be considered prior to screening of other family members for carrier status. If a deletion is suspected, it would also be appropriate to check for uniparental disomy and for a normal karyotype.
- *Detection of 1 pathogenic range GAA expansion and 1 frataxin mutation in an ataxic individual* - the pathogenicity of the "mutation" should be considered and commented on in the report. Testing of parents should be recommended in these cases to ensure that the "mutation" and the expansion are not present on the same allele. If the "mutation" tracks with the disease in the family it is most likely pathogenic and the compound heterozygote can be reported as diagnostic of FRDA. The implications for relatives of the patient should be commented on and carrier testing offered.
- *Detection of 1 pathogenic range GAA expansion and no other frataxin mutation in an ataxic individual* - the possibility of a missed mutation must be considered and reported on. If a suitable family structure exists, linkage analysis could be

performed. Since the carrier frequency in the population may be as high as 1 in 90, the presence of an expanded allele may not be related to the ataxia. The implications for relatives of the patient should be commented on and carrier testing offered.

- *Detection of 2 normal range GAA repeat alleles* - No confirmed FRDA patients with 2 point mutations (no GAA expansions) have been reported to date. If consanguinity is suspected, homozygosity for a frataxin mutation is a possibility. It is considered that homozygous inactivating mutations of frataxin may be lethal. Individuals with no GAA expansions should be reported with wording such as "diagnosis of FRDA unlikely". The possibility of the ataxia being as a result of one of the spinal cerebellar ataxias should be reported on and molecular testing offered, if appropriate.

As the carrier frequency is quite high in FRDA, carrier testing should be offered to spouses of known carriers and the risk to the couple of having an affected child estimated.

Reference sample for test optimisation and validation

A panel of FRDA reference cell lines is in the process of being prepared. When available, their source will be posted to this site.

It is envisaged that laboratories will be able to request reference DNA samples along with information about the mutations characterised in each sample in order to set up and optimise mutation detection techniques or samples "blinded" to mutation information in order to validate techniques. Prior to these reference samples being available, laboratories setting up FRDA testing are strongly advised to obtain positive control DNAs from a well established centre.

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Primer sequences

The primer sequences listed are taken from the references and web sites indicated. Individuals should consult the appropriate reference for full instructions and information on primer use. While every effort has been made to ensure the accuracy of the primer sequences, individual users should confirm these sequences before use.

GAA repeat amplification

Filla et al (1996)

- PCR product is (500 +3n)bp n= number of GAA repeats
- GAA-104F 5'-ggcttaaacctcccacacgtgt-3'
- GAA-629R 5'-AGGACCATCATGGCCACACTT-3'

Campuzano et al (1996)

- PCR product is (457 +3n)bp n= number of GAA repeats
- GAA-F 5'-gggattggtgcccagtgcttaaaagtag-3'
- GAA-R 5'-GATCTAAGGACCATCATGGCCACACTTGCC-3'

Campuzano et al (1996)

- Normal PCR product is 1.5kb. This primer pair are claimed to more efficiently amplify expanded alleles in heterozygotes
- Bam 5'-GGAGGGATCCGCTGGGCAAAGG-3'
- 2500F 5'-CAATCCAGGACAGTCAGGGCTTT-3'

Exon amplification

Campuzano et al (1996)

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- Exon 1 (240bp)
- 1F 5'-AGCACCCAGCGCTGGAGG-3'
- 1R 5'-CCGCGGCTGTCCCGG-3'
- Exon 2 (168bp)
- 2F 5'-AGTAACCTACTTCTTAACCTTTGGC-3'
- 2R 5'-AGAGGAAGATACCTATCACGTG-3'
- Exon 3 (227bp)
- 3F 5'-AAAATGGAAGCATTGGTAATCA-3'
- 3R 5'-AGTGAACATAAAATCTTAGAGGG-3'
- Exon 4 (250bp)
- 4F 5'-AAGCAATGATGACAAAGTCTAAC-3'
- 4R 5'-TGGTCCACAATGTCACATTCGG-3'
- Exon 5a (223bp)
- 5aF 5'-CTGAAGGGCTGTGCTGTGGA-3'
- 5aR 5'-TGTCCTTACAACGGGGCT-3'
- Exon 5B (224bp)
- 5bF 5'-cccatgctcaagacatactcc-3'
- 5bR 5'-acagtaaggaaaaacaacagcc-3'

Triplet repeat PCR (TP-PCR)

Schmitt et al (contact M Koenig for further details)

- P1 5'-GCTGGGATTACAGGCGCGCGA-3'
- P3 5'-TACGCATCCCAGTTTGAGACG-3'
- P4 5'-TACGCATCCCAGTTTGAGACGGAAGAAGAAGAAGAAGAA-3'

Linkage analysis

(Rodius et al 1994)

- FR1.PCR1.1 5'-CTAGAGTAGATAAGCTATACAT-3'
- FR1.PCR1.2 5'-TGGTGAATTACAGCTTTGACT-3'
- QZ105 (FR2) 5'-GATTGTGCCACTGCACTCCAAC-3'
- RA79 (FR2) 5'-CACATAATTTGTCATTTTATGTCTG-3'
- RA168 (FR7) 5'-ACCCCATGGGTTTCTCAAAT-3'
- RA169 (FR7) 5'-AATGCAAGTACACAAGCACACG-3'
- RB125 (FR8) 5'-GCACACTTACATACCTGGCTCT-3'
- RB126 (FR8) 5'-GTGAGTTAAGATTGTACCACTGCA-3'
- QQ244 (FR5) 5'-GGAGTTATAATCATCCCCTCTCA-3'
- QQ245 (FR5) 5'-CTCATCCATCCCTTCTGGCCT-3'

Montermini et al. 1995

- AFMb324xg1a 5'-CAACTTGGCAAAGGTAGGTG-3'
- AFMb324xg1m 5'-AGCTGGAGCCTCACACTG-3'
- AFMc003za5a 5'-CCTCAGTTTGTCTGGGACC-3'
- AFMc003za5m 5'-CGCATTCTTACAGTTGG-3'
- AFMc013yh5a 5'-CATGAGAGCACTGTATGAGGAC-3'
- AFMc013yh5m 5'-ACATCAGGATTGTGGGTTTC-3'

Useful www. resources

- A GeneCard entry exists for Frataxin (FRDA)
- Genome database Frataxin entry
- Friedreich Ataxia Association

General web resources

- Universal Mutation Database - gene/disease mutation databases
- Online Mendelian Inheritance in Man (OMIM)
- Genome database (GDB)
- Human gene mutation database (Cardiff)
- "Genecards"
- Human Genome Mapping Project Resources Centre
- European Directory of DNA Laboratories (EDDNL)



Table 1. Nomenclature and gene ID

OMIM #	OMIM Name	Gene Name	GDB ID
229300	Friedreich ataxia (FRDA) (FA)	Frataxin gene X25	Frataxin