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TCAGTACCGTACTTAG CLINICAL MOLECULAR ACGTGTC
GTGTCAGTACCGTACT GENETICS SOCIETY ACGTGTCAG

Practice Guidelines for the Molecular Diagnosis of Haemophilia A.

Guidelines prepared by Steve Keeney, Mike Mitchell and Anne Goodeve on behalf of the Clinical Molecular Genetics Society, the UK Haemophilia Centre Doctors' Organisation (UKHCDO) and the Haemophilia Genetics Laboratory Network following a workshop held on 9th October, 2003.

1.0 GENERAL RECOMMENDATIONS

It is recommended that genetic testing for haemophilia in the UK should preferably be performed in a member laboratory of the UKHCDO Haemophilia Genetics Laboratory Network. This is a consortium of laboratories, mostly within Comprehensive Care Haemophilia Centres, which work to agreed peer-reviewed standards of quality.

2.0 NOMENCLATURE AND GENE ID

Table 1.

Gene Name	Factor VIII
HUGO nomenclature	(F8)
OMIM Number	306700
GeneCards ID	F8
Ensembl Gene ID	ENSG00000165769
Chromosomal location	Xq28
Medline MESH Term	Haemophilia A, factor-VIII
NCBI LocusLink	HsF8 (Locus ID 2157)

3.0 DESCRIPTION OF THE DISEASE

Haemophilia A is a X-linked, recessively inherited bleeding disorder which results from deficiency of procoagulant factor VIII (FVIII). Affected males suffer from joint and muscle bleeds and easy bruising, the severity of which is closely correlated with the level of activity of coagulation factor VIII (FVIII:C) in their blood.
Haemophilia severity is defined by FVIII:C level in plasma, where severely affected individuals have <0.01 iu/dl (<1% of normal); moderate 0.01-0.05 iu/dl (1%-5% of normal); and mild >0.05 - <0.40 iu/dl (>5% - <40% of normal) (White et al, 2001). The disease affects approximately 1 in 5,000 males world-wide (reviewed in Forbes, 1997, Bolton-Maggs and Pasi, 2003).

3.0 COMMON REASONS FOR REFERRAL

Family history of the disease is an indicator for referral, however, approximately one third of cases have no prior history of haemophilia A (sporadic disease). In severe haemophilia A, diagnosis often follows the observation of unexplained severe bruising or bleeding in young males, who often present when they first become mobile around one year of age. Their haemophilia status can readily be assessed by measurement of plasma FVIII:C level. Where there is a prior family history of haemophilia, male cord blood can be tested at birth to determine FVIII:C. Males with moderate to mild haemophilia may not present until adult life. It is recommended that all children with haemophilia are investigated to establish the causative FVIII gene mutation. For detailed discussion of genetic service provision in inherited bleeding disorders, reference should be made to the UKHCDO document "Clinical Genetics Services for Haemophilia" (ISBN 901787 07 9).
Genetic analysis is required to reliably determine female carrier status, as Lyonisation can markedly skew female FVIII:C levels. Female relatives may request carrier analysis when a male relative is first diagnosed as having haemophilia, when they wish to start a family, or frequently, when in early pregnancy.
Genetic counselling should be performed by suitably qualified health professionals with in-depth knowledge of haemophilia. Ideally a professional with experience of managing and treating patients with haemophilia and their families should be involved.

4. 0 THE GENE

The factor VIII gene spans 186kb and is comprised of 26 exons, which range from 69bp (exon 5) to 3.1kb (exon 14) in size. The FVIII message is nearly 9kb in size and encodes a mature protein of 2332 amino acids. Mild/moderate haemophilia A and approximately half of all severe haemophilia A results from heterogeneous mutations which occur throughout the FVIII gene. For a review of the molecular aspects of haemophilia A see Bowen (2002).
A regularly updated website http://europium.csc.mrc.ac.uk/WebPages/Main/main.htm is maintained by Dr. Kemball-Cook at the MRC Clinical Sciences Centre, London.
Further tips can be found here http://europium.csc.mrc.ac.uk/WebPages/Database/Methods/methods.html

5.0 APPROACHES AND PROTOCOLS

5.1 Diagnostic strategy

The severity of haemophilia A in the pedigree should be determined first as this will influence the diagnostic strategy employed. Severe haemophiliacs should be screened for the intron 22 inversion mutation followed by the intron 1 inversion mutation. This approach should identify the underlying

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mutation in 45-50% of severe haemophilia A patients. The remaining severe haemophilia A pedigrees should then be analysed further either by full mutation or linkage analysis.

Moderate and mild haemophilia A is not associated with a common mutational mechanism and patients require either full mutation or linkage analysis.

5.1.1 Intron 22 inversion screening

The factor VIII gene intron 22 inversion mutation (Lakich et al, 1993; Naylor et al, 1993) accounts for disease in 20% of all patients and always produces severe disease (causative mutation in approximately 45% of severe haemophilia A). It results from homologous recombination between copies of a repeated DNA sequence, the intron 22 homologous region (int22h), one copy located in intron 22 of FVIII, the other two copies distal and telomeric to FVIII.

In families with severe haemophilia A, the affected male(s) should first be tested for the presence of the FVIII intron 22 gene inversion. The inversion is detectable by Southern blotting (Lakich et al, 1993) or more recently by Long PCR based protocols (Liu et al, 1999) (sections 5 and 12). The Long PCR method allows results to be obtained within 24 hours and uses a small amount of DNA, an important consideration when performing PND on a limited quantity of chorionic villus biopsy (CVB) material.

The second most common mutation in severe haemophilia A is the intron 1 inversion mutation. This was initially reported to be present in approximately 5% of patients (Bagnall et al, 2002) but in the UK severe haemophilia A population it was subsequently reported to have a frequency of 1.8% (Cumming, 2004).

Where no affected male is available, an obligate carrier female can be tested instead to determine the presence of an inversion mutation in the family.

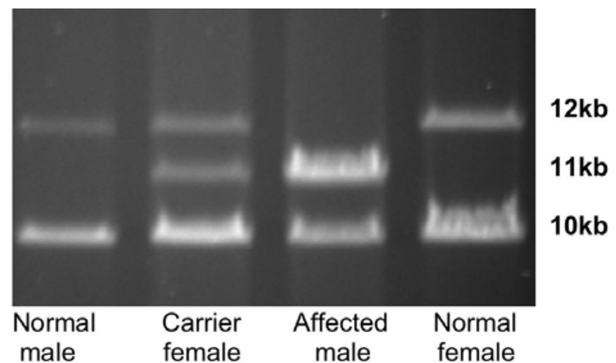
If the intron 1 or intron 22 inversion is present in a family, carrier status of any female relative can be readily determined.

Disease in the remaining severe, moderate and mild patients is predominantly due to point mutations, small insertions and deletions. Large deletions and insertions are rarer.

5.1.2.1 Intron 22 inversion detection by Long PCR:

Long PCR protocols for detection of the intron 22 inversion are now in common usage. This method for detection of the FVIII gene intron 22 inversion removes the requirement for Southern Blotting and results can be obtained within 24 hours. Modifications from standard long range PCR protocols include the addition of DMSO and incorporation of deaza dGTP to enable read through of a high GC content region of the FVIII gene. The method relies on multiplex PCR and generates a constant PCR product which appears in all templates. This band acts as a control to show that the reaction has worked efficiently. The largest amplification product seen using this method is 12 kb, well within the range of the long PCR DNA polymerase mix utilised. Establishing the method can prove technically demanding. The most informative reference for the standard method is Liu and Sommer, 1998. Of paramount importance is the quality of template DNA. Degraded or sheared DNA will not amplify. DNA quality can be monitored by electrophoresis, on a 1% agarose gel it should run with a size estimate of >50 kb. Success may also be achieved when using the described primers above but in modified protocols (Bowen and Keeney, 2003).

Figure 2. Long PCR of the FVIII intron 22 gene inversion.



Key: An upper 12 kb band and a lower 10 kb band indicate the inversion is not present.

A middle 11 kb band and a lower 10 kb band indicate the inversion is present in an affected male.

Bands at 10, 11 and 12 kb indicate a female carrier of the inversion.

5.1.2.2 Intron 22 inversion detection by Southern Blot

To detect the mutation by Southern Blot, 10µg genomic DNA is restricted with 15-20U *Bcl* I overnight at 50°C, electrophoresed, typically on an 0.6% agarose gel (1400Vhr) and probed with a 1.0kb *Eco* RI-*Sac* I fragment of p482.6 (section 12). The gel should clearly resolve fragments of 21.5, 20.0, 17.5, 16.0 15.5 and 14.0kb

The distal inversion (type 1) involves the copy of int22h furthest from FVIII, whereas the proximal inversion (type 2) involves the int22h copy closer to the FVIII gene. Occasional individuals have more than two extragenic copies of int22h and recombination can also occur with these (type 3), giving rise to more complex banding patterns (Antonarakis et al, 1995). Care should be taken when interpreting abnormal patterns. Females with the inversion mutation are heterozygous carriers of severe haemophilia A.

The inversion can also be detected indirectly in males by RT-PCR as a lack of message across exon 22-23 of FVIII, but female carrier status cannot be determined by this method. It is recommended that confirmation of inversion status in any male diagnosed by this process be obtained by a direct assay method.

5.1.3 Factor VIII Gene Intron 1 Inversion PCR amplification

This inversion splits the FVIII gene at intron 1 producing 2 chimeric mRNAs. One of these mRNAs contains the first exon of the FVIII gene followed by exons 2 to 6 of the *VBP1* gene coding for the subunit 3 of prefoldin. The other mRNA contains all but the last exon of the *C6.1A* gene followed by exons 2 to 26 of the FVIII gene. The breakpoint regions have been characterised and a dual PCR assay has been devised for the detection of this mutation (Bagnall et al, 2002).

Both assays are designed to amplify independently sequence flanking the int1h regions (from intron 1 homologous sites). *Int1h-1* specifies the assay for the copy in the FVIII gene and *int1h-2* the homologous region 140 kb more telomeric (section 9).

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This assay can be performed by standard PCR and is robust. However, there has been a report of partial gene deletions or int1h duplications which may give abnormal or unexpected banding patterns (Vinciguerra et al, 2003) therefore it is recommended that the assay should always be carried out for both int1h-1 and int1h-2 to reveal these anomalies.
Since this mutation is the second most common described it should be sought in all families with severe haemophilia A during initial genetic diagnosis.

5.2 Mutation Detection Strategies

5.2.1 Previously Characterised Mutations

Many laboratories are now screening their patients for mutations in the FVIII gene (see the
UKHCDO Directory of Molecular Diagnostic Services for Inherited Bleeding Disorders,(http://www.ukhcdo.org/). The UKHCDO haemophilia patient database, an annually updated reference to all UK registered patients, notes whether a mutation has been detected in a particular patient. Details of the mutation characterised in a patient of interest are only available from their Haemophilia Centre Director (see listing on UK Haemophilia Society website).

5.2.2 Unknown Mutation Detection

Mutations are generally sought in affected males and then confirmed or excluded in female relatives. The method selected will be dependent on resources and expertise available in a particular laboratory. Current methods which have been applied by many centres performing mutation pre-screening in the UK haemophilia A population rely on heteroduplex formation and subsequent detection of mismatched heteroduplexes. There are two major heteroduplex formation methods in current use in the UK; conformation sensitive gel electrophoresis (CSGE) and denaturing high performance liquid chromatography (dHPLC).

5.2.3 CSGE

Conformation sensitive gel electrophoresis (Ganguly et al, 1993) is a variant of heteroduplex analysis which has been applied to screening the factor VIII gene for mutations (Williams et al, 1998). It has the advantages of being simple and relatively rapid to perform and does not require the use of radiolabel. Despite this apparent simplicity, the technique requires a great deal of skill, both technical and interpretive, to achieve good sensitivity. PCR products subjected to CSGE should optimally be no greater than 500 bp and have a high degree of overlap (> than 100bp is desirable) when amplifying large exons such as 14 and 26. Given careful design and application an expected mutation detection sensitivity of >90% can be expected.

5.2.4 dHPLC

Denaturing high performance liquid chromatography (Oefner et al, 1995) separates hetero- and homoduplexes due to their differences in melting behaviour and subsequent retention time on a non-porous polystyrene-divinylbenzene matrix. DHPLC requires specialist equipment (most commonly used is the Transgenomic Wave System) but is otherwise a technically straightforward and rapid way to screen for mutations in the FVIII gene (Oldenburg et al, 2001).
Care should be taken with assay design. Good primer design, assisted by software analysis of amplicon melting characteristics, is essential if high detection sensitivity is to be achieved.
An experienced scientist should expect > 95% sensitivity (150-500 bp). Fragments less than 110-120 bp in size are liable to loss of detection sensitivity. Larger fragments (up to 1-1.2 kb) can be studied but this invariably results in the need to accommodate more melt domains and hence more injections per sample at a range of oven temperatures.
Best Practice Guidelines have been produced for dHPLC and can be found on the Clinical Molecular Genetics Society (CMGS) website.
Other mutation pre screening methods may be used but are not currently employed by the Network.

5.2.5 Direct DNA Sequencing

DNA sequencing is the gold standard for mutation detection in DNA from males. In the case of female carriers, heterozygosity for a mutation may not be readily detected with some sequencing approaches. Using streamlined procedures the essential regions of the FVIII gene are now amenable to direct DNA sequence analysis in a rapid and cost effective fashion, given the appropriate infrastructure. Streamlined methods, including automated or semi-automated procedures can generate full sequence data for the FVIII gene within the rapid timescale often required in a diagnostic setting. Normally a candidate mutation would be identified in a hemizygous male haemophiliac before applying DNA sequencing to determine the presence or absence of a nucleotide alteration in at risk family members. Failing this, a known obligate carrier female can be used for initial mutation identification, with the reservation noted above.

5.3 DNA Sequencing Best Practice

Refer to the CMGS Sequencing Best Practice Guidelines for guidance on minimum sequence quality and interpretation standards. It is recommended that that the following points be given particular attention: Software analysis tools (e.g. tools which facilitate comparative sequence analysis such as the Staden Package) should be employed when analysing large quantities of DNA sequence data. Sequence analysis should always be performed on both forward and reverse strands.
Any sequence change used for diagnosis should be confirmed by repeat sequencing in relevant family members. As a minimum this should be done with recourse to the original DNA (or stored blood) sample and reamplification from the original sample. Some centres may wish to issue an interim report until they have been able to verify a base change in an independent sample from relevant individuals.

5.4 Mutation Validation

When a novel nucleotide change is found, caution should be exercised before deciding that it is the one responsible for disease. The entire FVIII gene should be analysed for sequence alterations. Whereas termination, deletion and insertion mutations may obviously be causative, missense and other changes may not. The haemophilia A database (HAMSTeRS) should be consulted to determine whether the change has been previously reported. Entries on the HAMSTeRS FVIII mutation database need to be interpreted with a degree of caution. Minimum checks should include the following questions:

- Does the reported severity agree with the FVIII level in the patient being analysed?

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- Has the candidate mutation been reported previously as a polymorphism?
- Could the candidate mutation affect splicing? Software tools, e.g. StrataSplice or the NetGene2 server can be used to allow alternative splice site prediction.
- For missense mutations, does the nature and location of the amino acid substitution confer a high risk of being detrimental to protein structure/function?
- Is the changed amino acid conserved across species (see http://europium.csc.mrc.ac.uk/WebPages/Database/Protein/lineups.html)?
- Could the ethnic origin of the patient affect interpretation of polymorphism/candidate mutation status for a given base change?

Where uncertainty remains the family should be tested to determine whether the nucleotide alteration tracks with the disease. Further corroboration may be obtained by genotyping a panel of normal DNA samples of the same ethnic origin, where available, to rule out a polymorphic change. Wherever possible candidate mutations should be confirmed in affected and excluded in unaffected males on the maternal side of the family. Candidate mutations can still be used as bespoke genetic markers if they track appropriately within the family, irrespective of their disease association.

Although DNA sequencing of the essential regions of the FVIII gene should have a very high degree of sensitivity, there remains the possibility that a proportion of patients will have a mutation which lies outwith the regions being analysed. Data suggest that current DNA sequencing strategies will detect mutations or candidate mutations in 98% of haemophilia A males (Klopp et al, 2002).

5.5 Linkage Analysis

Historically, linkage analysis was the method most commonly used to determine female carrier status in families with haemophilia A. Linkage studies are being superseded by direct mutation analysis protocols (see sections 5.1 and 5.2). However, intragenic linked markers are still useful and may be of particular value under certain circumstances, such as:

- Where a family has previously been investigated by linked markers and the mutation has not been identified
- Where a mutation has not been verified
- Where a mutation has not been found

Only intragenic markers should be considered for use. There are two dinucleotide repeats and several dimorphisms within the FVIII gene. When three or four markers are used in combination, carrier status can be determined in approximately 80% of affected families. In those families with sporadic haemophilia (one third of families), female relatives can only be excluded from being carriers where they do not share an allele with the affected male.

5.5.1 Dinucleotide Repeats

Dinucleotide repeats in introns 13 and 22 (Lalloz et al, 1991, 1994) are generally the first choice of markers as they have the highest rates of heterozygosity. A wide variety of methods can be used for their analysis; the method selected will depend upon laboratory circumstances. The simplest method involves PCR amplification followed by electrophoresis on a native polyacrylamide gel, and detection by ethidium bromide staining. Silver staining obviates the need for darkroom facilities. Alternatively, one primer can be end labelled with 32P, and labelled PCR products detected following electrophoresis on a denaturing polyacrylamide gel. This method is tedious, but effective at allele discrimination. For fluorescent detection, one primer is end fluorescent labelled, product is detected following electrophoresis on a gel in an automated sequencer used for genotyping (Noble et al, 1996). This method has the advantage of automation, but may be expensive to run. All analysis of the dinucleotide repeats suffers to an extent from stutter bands which occur due to polymerase slippage during PCR amplification. These additional bands can complicate allele size identification.

The possibility exists that expansion or contraction of these dinucleotide repeats may occur between generations and this should be recognised.

5.5.2 Dimorphisms- Standard PCR Analysis

Commonly used dimorphisms in the FVIII gene which can be analysed by PCR include those detected by digestion with *Bcl* I in intron 18 (Kogan et al, 1987), *Hind* III in intron 19 (Graham et al, 1990), and G/A in intron 7, which can be detected using an introduced *Alw* NI site (Kogan & Gitschier, 1990). Of these, *Bcl* I is the most widely used. *Hind* III is in strong linkage disequilibrium with *Bcl* I, so only one of these two markers should be analysed. The fragment amplified for *Hind* III dimorphism analysis also contains a constant *Hind* III restriction enzyme site. A further dimorphism within intron 22, *Msp* A1I has been described which, despite close proximity to the *Xba* I dimorphism, is not in complete linkage disequilibrium and may be useful in some family studies (Bowen et al, 2000).

5.5.3 Dimorphisms- Southern Blot and Long PCR Analysis

The *Xba* I dimorphism in intron 22 is detectable by Southern blotting (section 5.1.2b) or more recently by Long PCR (El-Maarri et al, 1999; De Brasi et al, 1999) as it lies in the int22h repeated DNA region. Most ethnic groups have a heterozygosity rate of close to 0.5. There is also an *Xba* I polymorphic site in the two extragenic copies of the int22h region, with a heterozygosity rate in Caucasians of about 0.1. As the extragenic copies of int22h are telomeric to the FVIII locus by ~500kb, the recombination rate between them and the FVIII gene should be less than 1%.

To detect the polymorphism by Southern blot, digest genomic DNA with 15U each of *Xba* I and *Kpn* I, or *Asp* 718 (isoschisomer of *Kpn* I), overnight at 37°C, and electrophorese on an 0.8% agarose gel ~ 1200Vhr. Probe with the 1.0kb *Eco* RI / *Sac* I fragment of p482.6. This is the same probe as is used for the intron 22 gene inversion. The bands detected are given in table 2:

Table 2. See text.

Location	Site Absent	Site Present
Extragenic	6.8 kb	5.4 kb
Extragenic	6.6 kb	5.2 kb
Intragenic	6.2 kb	4.8 kb

5.5.4 Extragenic Polymorphisms

Markers at St14 and DX13 were the first reported markers for FVIII gene tracking. However, their distance from the FVIII gene results in a risk of recombination of 5% per meiosis. For this reason extragenic markers should not be used. Where demonstration of linkage using intragenic markers fails the use of mutation screening is indicated.

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5.5.5 Linkage Analysis Problems

Linkage analysis fails in a number of families for one of the following reasons;

- Lack of prior family history (see section 3 above)
- Key pedigree members not available.
- Polymorphisms uninformative in key female(s).
- Non-paternity

In these families, mutation detection should be used. Linkage analysis cannot determine the carrier status of the mother of a haemophiliac.

Wherever possible, mutation detection should be used for genetic counselling in haemophilia A families. However, this is not always practicable, for example, the timescale for mutation characterisation can be several weeks and this is clearly inappropriate for a pregnant female who desires carrier status determination or prenatal diagnosis and the causative mutation in the family is not known. Where direct mutation detection is not feasible, linkage analysis provides an acceptable alternative which offers a high degree of diagnostic confidence.

6.0 PRENATAL DIAGNOSIS

Prenatal diagnosis is generally performed by chorionic villus sampling at between 11 and 13 weeks of gestation. Direct karyotype analysis can be performed to determine foetal sex and to ensure that there are no chromosomal abnormalities. Rapid PCR based sexing protocols using amelogenin (AMXY) specific primer sets are in common usage. Female foetuses sexed by this method require confirmation that no maternal contamination is present in the sample. Female foetuses require no further analysis. Their haemophilia carrier status should be determined later in life.

Male haemophilia status can be determined by analysis of a previously determined familial mutation or informative marker. For analyses which involve PCR amplification, results should be provided within 2-3 days of the CVS sample being taken, where Southern blotting is required, the analysis may take up to 10 days to complete.

More detailed discussion on general issues relating to prenatal diagnosis can be found in the UKHCDO document "Clinical Genetics Services for Haemophilia" (ISBN 901787 07 9).

7.0 WORDING OF REPORTS

Reports must be clear, concise, accurate, fully interpretive, credible and authoritative. For general guidance on report writing refer to the CMGS Report Writing Best Practice Guidelines.

7.1 Linkage analysis using intragenic markers

The following wording is suggested;

The female can be diagnosed as a carrier/excluded from being a carrier, with a risk of error due to meiotic recombination of <1%.

7.2 Mutation Analysis Reporting

Wording will depend on the confidence placed in the interpretation of any candidate mutation, as discussed in section 5.4. Suggested wording for a mutation which has a high confidence attached to it may include**:**

Mutation analysis in males

"x has a mutation (No.nt>nt, aaNo.aa), previously reported in the FVIII gene/ not previously reported. The mutation is consistent with the severity of haemophilia A in x".

A brief explanation as to why a novel mutation is considered causative should be included, especially for a missense mutation. For example, the altered amino acid is conserved across X (a number of) species, and/or is structurally or functionally important; this base change has been excluded as a common polymorphism by analysis of >100 normal alleles; etc.

Mutation analysis in females

"y carries a FVIII mutation (No.nt>nt, aaNo.aa) which is consistent with the severity of haemophilia A seen in male relative x.

8.0 MUTATION ANALYSIS NOMENCLATURE

For guidance on nomenclature conventions refer to the CMGS sequencing best practice guidelines where the recommendations of the Human Genome Variation Society http://www.genomic.unimelb.edu.au/mdi/mutnomen/ are suggested for mutation reporting. The intron or exon containing the mutation should be stated. Note that in the absence of a complete genomic sequence for the FVIII gene that intronic numbering should be given as +/- with respect to the relevant exon, that is:

- *"-" for intronic mutations upstream of an exon, where "-1" is the intronic nucleotide immediately 5' to the first exonic nucleotide;*
- *"+" for intronic mutations downstream of an exon end, where "+1" is the intronic nucleotide immediately 3' to the last exonic nucleotide.*
- The use of lower case letters for intronic sequence is recommended to avoid confusion with cDNA numbering.
- The numbering convention used in the HAMSTeRS database should be followed:
- For cDNA nucleotide numbering +1 is the first base of the initiator methionine codon.
- Amino acid numbering starts at +1 for the first codon of the mature protein. The signal peptide is numbered from -19 (initiator methionine) to -1 (serine).
- To avoid potential confusion between single letter amino acid codes and nucleotides the following convention is recommended: *Nucleotide position 5822 A>G, corresponding amino acid postion N1992S.*
- It is recommended that in the body of the report the full name of each amino acid is specified to avoid confusion between single letter amino acid and nucleotide codes, e.g. "*Cysteine (C) 1234 to Alanine (A), or C1234A*".

9.0 DETECTION OF LARGE SCALE DELETIONS IN HETEROZYGOUS CARRIERS

Large deletion mutations are readily detected in affected males due to lack of amplification of missing regions of the FVIII gene. It is particularly difficult to detect these mutations in female relatives, where the failure to amplify one of the two FVIII alleles must be detected. Possible methods for detection of female carrier status in these families include;

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- Use of linkage analysis, which may reveal loss of heterozygosity for markers in the deleted region.
- Methods based on gene dosage analysis may be utilized
- Gap PCR protocols may be developed where deletion boundaries are known
- Other methods, such as Multiplex Ligation-dependent Probe Amplification (MLPA), could be applied to the FVIII gene.

None of the latter three methods are in routine diagnostic use and may need to be developed specifically for a given investigation.

10.0 MOSAICISM

Germline and somatic mosaicism may complicate any genetic diagnosis in haemophilia A. Particular attention should be given to the possibility of mosaicism in sporadic haemophilia where the mother of an affected male does not appear to carry the mutation in her leucocyte DNA, particularly where the apparently *de novo* mutation is a point mutation (Leuer et al, 2001).

It is recommended not to state that the mother of a haemophiliac is not a carrier, even when the mutation is not identified in her somatic DNA. A specific reference to the possibility of germline mosaicism may be added.

11. VON WILLEBRAND FACTOR - FACTOR VIII BINDING ANALYSIS

A number of patients with von Willebrand disease (VWD) have been previously misclassified as having mild haemophilia A. This results from their VWF having a defect in its FVIII binding site (type 2N VWD), the resulting phenotype in a homozygous or compound heterozygous individual mimicking mild haemophilia A. The VWF gene is autosomally inherited. The two disorders can be discriminated by an ELISA based FVIII binding assay, which determines the FVIII binding capacity of patient's VWF. Some laboratories use this assay to examine all mild haemophilia A and VWD patients prior to their genetic analysis. Alternatively, it may be used where the FVIII deficiency does not show clear X-linked inheritance.

12. REFERENCE SAMPLES FOR TEST OPTIMISATION AND VALIDATION

An EQA scheme has been established for haemophilia A genetic investigation. Details are available from UK NEQAS (Coagulation). Participation in EQA is recommended.

There are currently no commercially available reference materials for FVIII gene analysis. These may become available in the future through the NIBSC

13.0 LABORATORY MATERIALS

13.1 Gene Inversion PCR

For primer sequences see table 3.

13.2 Intragenic marker PCR

For primer sequences see table 4.

Table 3. FVIII gene intron 22 gene inversion long PCR and intron 1 inversion PCR

Primer designation	Sequence 5'-3'	PCR product size	Reference
	Intron 22 long PCR		
INT22 P	GCCCTGCCTGTCCATTACACTGATGACATTATGCTGAC	10 , 11, 12kb	Liu and Sommer, 1998
INT22 Q	GGCCCTACAACCATTCTGCCTTTCACTTTCAGTGCAATA		
INT22 A	CACAAGGGGGAAGAGTGTGAGGGTGTGGGATAAGAA		
INT22 B	CCCCAAACTATAACCAGCACCTTGAACTTCCCCTCTCATA		
	Intron 1 inversion PCR – Fragment 1: Int1h-1	1.5, 2kb	Bagnall et al, 2002
9F Int1h-2F	GTT GTT GGG AAT GGT TAC GG GGC AGG GAT CTT GTT GGT AAA CTA GCT		
9cR	TGA GCT CCC TGT GG		
	Intron 1 inversion PCR – Fragment 2: Int1h-2	1.5, 2kb	Bagnall et al, 2002
9F Int1h-2F	GTT GTT GGG AAT GGT TAC GG GGC AGG GAT CTT GTT GGT AAA TGG GTG		
Int1h-2R	ATA TAA GCT GCT GAG CTA		

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Table 4. Intregenic marker primer sequences.

Primer designation	Sequence 5'-3'	PCR product size/bp*	Reference
"*Alw* NI" forward "*Alw* NI" reverse	TAA TGT ACC CAA GTT TTA GG TAT AGA ACA GCC TAA TAT AGC AAC AGA CTC	260bp (-)232 & 28bp (+)	Kogan & Gitschier, 1990
Intron 13 forward Intron 13 reverse	TGC ATC ACT GTA CAT ATG TAT CTT CCA AAT TAC ATA TGA ATA AGC C	(CA)n 20 repeats = 141bp	Lalloz et al, 1991
Bcl I forward *Bcl* I reverse	TAA AAG CTT TAA ATG GTC TAG GC TTC GAA TTC TGA AAT TAT CTT GTT C	142bp (-) 99 & 43bp (+)	Kogan et al, 1987
Hind III forward Hind III reverse	AAG GTC CTC GAG GGC GAG CAT AAG GTC GGA TCC GTC CAG AAG	Product = 717bp 469 & 248bp (-), 469 & 167 & 81bp (+)	Graham et al, 1990
Intron 22 forward Intron 22 reverse	TTC TAA GAA TGT AGT GTG TG TAA TGC CCA CAT TAT AGA	(GT)n (AG)n 26 repeats = 83bp	Lalloz et al, 1994
*Xba*I Intragenic: Forward XEF Reverse XER	 CTGGAGAATCTAAGAGGATAGAGGACAACATTTACC AGTACTTCTCCAGGGTCTGGGCGTGCTC	Product 6.6kb; (-) 6.1+ 0.5 kb, (+) 1.3+4.8+0.5 kb	De Brasi et al, 1999
*Msp*1 Intragenic: Nested PCR, first round *Xba*I Intragenic PCR above, second round: DWF Forward DWR Reverse	 GGTGCTCAGTAGCCTGTCGTTGTG GCCACTACGCTCAGGTCCTGAGTC	Product 176 bp; (-) 141+35 bp (+) 96+45+35 bp	Bowen DJ et al, 2000

Key: * + Indicates presence of restriction site, - indicates absence. For Caucasian allele frequencies refer to the Listing of Known Polymorphisms. See Goodeve (1998) or Goodeve & Peake (1997) for other ethnic groups.

13.3 Source of Probe

Plasmid p482.6 comprises pUC8 with a 9.6kb *Eco* RI insert from intron 22 of FVIII. This has homology with part of the int22h sequence. Digestion with *Eco* RI plus *Sac* I yields three fragments of 1.0kb ("probe a", Wion et al, 1986), used to probe Southern blots both for the intron 22 inversion and *Xba* I polymorphism, 8.6kb (intron 22) and 2.7kb (vector). The plasmid can be obtained from the ATCC code 57202, 57203

14.0 SPECIALIST REFERRAL LABORATORIES

A listing of services available for haemophilia A and B testing offered by UK laboratories is found in the UKHCDO Directory of Molecular Diagnostic Services for Inherited Bleeding Disorders (http://www.ukhcdo.org/)..

15. WEB RESOURCES

The Haemophilia A Mutation, Structure, Test and Resource Site (HAMSTeRS) has mutation and polymorphism databases for FVIII in addition to a partial molecular model of FVIII, methods pages and useful links to other sites.

UK Haemophilia Society lists all UK Haemophilia Centres, with Directors names and addresses.

World Federation for Hemophilia.

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