Practice Guidelines for the Molecular Diagnosis of Haemophilia A.

Guidelines prepared by Steve Keeney, Mike Mitchell and Anne Goodeve on behalf of the UK Haemophilia Centre Doctors' Organisation (UKHCDO), the Haemophilia Genetics Laboratory Network and the Clinical Molecular Genetics Society. All network members contributed to amending and approving the text. UPDATED July 2010

1.0 GENERAL RECOMMENDATIONS

Where guidance states this is *recommended* practice or text is given in italics then this should be followed to ensure quality of service.

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

Retention and storage of records and specimens relating to molecular diagnosis in the UK must conform with the Human Tissue Act 2004 and guidance from the Royal College of Pathologists and Institute of Biomedical Science on the retention and storage of pathological records and specimens, 4th edition, 2009.

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 DISORDER

Haemophilia A is an 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 <1 IU/dL (<1% of normal); moderate 1-5 IU/dL (1%-5% of normal); and mild >5 - <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).

3.1 COMMON REASONS FOR REFERRAL

Family history of the disease is an indicator for referral, however, approximately one third of cases have no prior family history of haemophilia A (sporadic disease) (Miller et al 1987, Kasper et al 2007). In severe haemophilia A, diagnosis frequently 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 (F8) 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 or by a team including a genetic counsellor and haemostasis specialist. A professional with experience of managing and treating patients with haemophilia and their families should be involved.

4.0 THE GENE

F8 spans 186kb and comprises 26 exons, which range from 69bp (exon 5) to 3.1kb (exon 14) in size. Key reference sequence identifiers are given in table 2.

Table 2.

Type of sequence	NCBI RefSeq accession number
RNA (cDNA)	NM_000132.x
Protein	NP_000123.x
Genomic DNA	NG_005114.x

(.x denotes version number)

The *F8* 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 *F8*. For reviews of the molecular aspects of haemophilia A see Pruthi 2005, Goodeve 2008.

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.

It is recommended that severe haemophiliacs should be screened for the F8 intron 22 inversion mutation followed by the F8 intron 1 inversion mutation. This approach should identify the underlying mutation in 45-50% of severe haemophilia A patients.

The remaining severe haemophilia A pedigrees should then be analysed further by full mutation analysis of F8.

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

5.1.1 Intron 22 inversion screening

The *F8* 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 *F8* (int22h-1), the other two copies (int22h-2 and int22h-3), distal and telomeric to *F8*.

In families with severe haemophilia A, the affected male(s) should first be tested for the presence of the *F8* intron 22 gene inversion. The inversion is detectable by Southern blotting (Lakich et al 1993) or more recently by Long PCR or inverse PCR based protocols (Liu et al 1998; Rossetti et al 2005 & 2008). The Long PCR method allows results to be obtained within 24 hours and uses a small amount of DNA, an important consideration when performing pre natal diagnosis (PND) on a limited quantity of chorionic villus biopsy (CVB) material. This method for detection of the intron 22 inversion can be technically demanding. Alternative methods based on inverse PCR may prove more reliable (see section 5.1.2.2).

The distal inversion (type 1) involves the copy of int22h furthest from *F8*, whereas the proximal inversion (type 2) involves the int22h copy closer to the 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 on Southern blotting (Antonarakis et al 1995). Care should be taken when interpreting abnormal patterns. Females heterozygous for the inversion mutation are carriers of severe haemophilia A. A detailed description of the arrangement of the int22h sequences is given in Bagnall et al 2006.

The second most common mutation in severe haemophilia A is the *F8* 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 found 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 have been used widely. This method for detection of the *F8* intron 22 inversion enables results to be obtained within 24 hours. Modifications from standard long range PCR protocols include the addition of DMSO to enable read through of a high GC content region of *F8*. 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. However, the original protocol based on the use of the Roche Expand Long Template PCR System® has been superseded by alternative protocols due to difficulties encountered when the kit was reformulated. 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 standard primers but in modified protocols (Bowen and Keeney 2003).

5.1.2.2 Intron 22 inversion detection by inverse PCR

An inverse PCR method, (Rossetti et al 2005) although appearing to be more complex and time-consuming than Long PCR has proved to be reliable in a diagnostic setting. It comprises *Bcl*I restriction enzyme digestion of genomic DNA followed by ring ligation to create templates for inverse PCR. Products are then visualised by standard gel electrophoresis. A more recent variant, inverse-shifting PCR enables detection of both proximal and distal inversions, plus insertions and deletions resulting from additional int22h recombination events (Rossetti et al 2008).

5.1.2.3 Intron 22 inversion detection by Southern blot

To detect the mutation by Southern blot, genomic DNA is restricted with *Bcl*I, electrophoresed, blotted onto a membrane and probed with an intron 22 sequence derived from plasmid p482.6. The gel should clearly resolve fragments of 21.5, 20.0, 17.5, 16.0 15.5 and 14.0kb identifying normal and mutated sequence. The plasmid p482.6 can be obtained from the ATCC, code 57202, 57203.

The inversion can also be detected indirectly in males by RT-PCR as a lack of message across exon 22-23 of *F8*, 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 *F8* intron 1 inversion PCR amplification

This inversion splits *F8* at intron 1 producing 2 chimeric mRNAs. One of these mRNAs contains the first exon of *F8*

followed by exons 2 to 6 of the *VBP1* gene coding for subunit 3 of prefoldin. The other mRNA contains all but the last exon of the *BRCC3* gene followed by exons 2 to 26 of *F8*. 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 (intron 1 homologous sites). Int1h-1 specifies the assay for the copy in *F8* and int1h-2 the homologous region 140 kb more telomeric (section 9).
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.
A method based on inverse-shifting PCR uses the same ring ligation template and amplification conditions as that for the intron 22 inversion method (Rossetti et al 2008). This approach allows analysis of both inversions within the same protocol.

5.2 Mutation detection strategies

5.2.1 Previously characterised mutations
All UK laboratories are now screening their patients for mutations in *F8*; details are given in the UKHCDO Directory of Molecular Diagnostic Services for Inherited Bleeding Disorders. 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). A regularly updated website, the Haemophilia A Mutation, Structure, Test and Resource Site (HAMSTeRS, now known as HADB, section 14), which includes a database containing reported mutations in *F8*, is maintained by Dr. Kemball-Cook at the MRC Clinical Sciences Centre, London (Kemball-Cook et al 1998). The human gene mutation database (HGMD) also acts as a repository for collated mutation data, but unlike HAMSTeRS, gives no indication of mutation frequency. Links to all of the above resources can be found in section 14, Web Resources.

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. Most laboratories in the UK now perform direct DNA sequencing of the essential regions of *F8* with no pre-screening step. However, some centres perform mutation pre-screening which relies on heteroduplex formation and subsequent detection of mismatched heteroduplexes by High Resolution Melt analysis (HRM).

5.2.3 HRM
High-resolution melting curve analysis (HRM) is a closed-tube fluorescent technique that can be used for genotyping and heteroduplex detection after initial PCR. The sensitivity and specificity of the technique depends on the length of amplicons used but a sensitivity of 93% has been reported when applied to haemophilia A mutation analysis (Lin et al 2008).
Where HRM fails to detect a candidate mutation it is recommended to proceed to direct DNA sequencing of the essential regions of F8 (see sections 5.2.4 and 5.4).

Other mutation pre-screening methods may be used but are not currently employed by the Network. For a review of these see Goodeve 2008.

5.2.4 Direct DNA sequencing
DNA sequencing is the gold standard for mutation detection in DNA from males, or in obligate carrier females where an affected male is not available. Using streamlined procedures the essential regions of *F8* 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 *F8* 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.
Sequence analysis should cover the essential regions of *F8* (the promoter region plus all exons and intron/exon boundaries, although some centres currently employ a non-standardised targeted exon screening approach. Mutation databases should be consulted to determine the extent of non-coding regions of *F8* that require analysis.
It is recommended to state on the diagnostic report which regions of F8 have been screened.

5.3 DNA sequencing best practice
Refer to the CMGS Sequencing Best Practice Guidelines for guidance on minimum sequence quality and interpretation standards (section 14). It is recommended that the following points be given particular attention: Software analysis tools (e.g. tools which facilitate comparative sequence analysis such as the Staden Package, section 14) should be employed when analysing large quantities of DNA sequence data. Sequence analysis should always be performed only on data of high quality. Unidirectional screening is now generally accepted as adequate for gene screening (Ellard et al 2009) but it is recommended that carrier status sequencing should be performed in both a 5' (Forward) and 3' (Reverse) direction.
In line with CMGS guidance, in the absence of a robust tube transfer checking system to assure sample identity at each stage (e.g. barcode scanning, witnessed transfers), mutations identified for the first time in a given pedigree should be confirmed by a second test, using a fresh template. Testing for known familial mutations should include, where possible, the use of a family member as the positive control.
In the case of PND it is recommended to confirm the identity of the sequence change in a sample from the mother. This may be from an archive sample or, where local quality control measures dictate, repeat analysis of an independent sample.
It is recommended that in cases where only the familial mutation is tested for that this is stated on the report.

5.4 Primer validation
Polymorphic variation may lead to allelic drop-out if a single nucleotide polymorphism (SNP) lies under a PCR primer, affecting its amplification efficiency. Since this possibility

cannot be eliminated completely it should be borne in mind when screening query carrier females who prove negative.
It is recommended to screen primer sequences for the presence of underlying polymorphism(s) when first designed, using an appropriate checking tool such as SNPCheck, and then when each new build of the SNP database (dbSNP) becomes available (section 14).
A judgement can then be made on the need to redesign a specific primer pair based on reported allele frequency data relating to the described polymorphic variation.

5.5 Mutation validation
When a novel nucleotide change is found (known as an unclassified variant, UV), caution should be exercised before deciding that it is the one responsible for disease (see below). The entire gene should be analysed for sequence alterations. Whereas termination, deletion and insertion mutations may obviously be causative, missense and other changes may not.
The UK and Dutch molecular genetic societies have produced a practice guideline for the interpretation and reporting of unclassified variants (sequence alterations of unknown pathogenicity, UV Guideline – see CMGS guideline link in section 14). A standard questionnaire is used to systematically evaluate and record the likely pathogenicity of the variant, assessing whether or not the variant is predicted to affect protein function, principally by means of cross-species amino acid alignment algorithms, evaluation using splice-site prediction tools and documented searches to demonstrate if the variant has been previously reported in the literature or on mutation databases. Note that some of the requirements of the UV guideline may be more rigorous than that required for a sex-linked recessive disorder where no other sequence change has been found in the gene of an index male. In addition there are *F8* specific resources which can be drawn upon when interpreting the significance, or otherwise, of a sequence variant. The haemophilia A database (HADB) and other available resources such as HGMD should be consulted to determine whether the change has been previously reported. Note that entries on mutation databases need to be interpreted with a degree of caution as they may not have been peer reviewed. Minimum checks should include the following questions:

- Is the previously reported disease phenotype consistent with that in the patient being investigated?
- Has the candidate mutation been reported previously as a polymorphism? This should be investigated utilising bioinformatics resources such as the list of polymorphisms given on HADB, the NCBI Single Nucleotide Polymorphism database (dbSNP, section 14) and published studies such as the resequencing of a panel of non-haemophiliac individuals from several population groups (Viel et al 2007).
- Is the changed amino acid conserved across species? At present there is no standard cross species alignment of the FVIII protein available as a resource for this purpose and laboratories are advised to refer to the UV Guidelines for advice when using this approach. A cross-mammalian alignment is available on the HADB database but this does not meet the criteria detailed in the UV guidelines.
- Could the candidate mutation affect splicing? The UV Guideline recommends that at least four *in silico* predictions are run before making any conclusion on pathogenicity.
- For missense mutations, does the nature and location of the amino acid substitution confer a high risk of being detrimental to protein structure/function? Interpretation of the significance of a DNA base change, particularly amino acid substitutions, should draw upon all information resources available, including interpretation of FVIII life cycle and structure/ function data. Structural data on FVIII is available in the HADB resources section. Prediction software, such as that referred to in the UV Guideline, can be used to investigate the likely effect of each amino acid substitution.

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 in order to minimise the risk that a rare polymorphism has been identified in a query carrier female (Schneppenheim et al 2009). Candidate mutations can still be used as bespoke genetic markers if they track appropriately within the family, irrespective of their disease association, although the possibility of non-linkage of the observed phenotype to the gene should be considered.
Rare cases have more than one candidate mutation identified in *F8* (0.4% on HAMSTeRS). Particular care should be taken in the interpretation of each variant and its possible contribution to the patient's phenotype.
Although DNA sequencing of the essential regions of *F8* 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 severe haemophilia A males (Klopp et al 2002). However, mutations have not been identified in an estimated 2-18% of haemophilia A patients overall (Oldenburg et al 2001, Jayandharan et al 2005, Vinciguerra et al 2006, Bogdanova et al 2007, Santacroce et al 2008) after exclusion of inversion events and mutations in the essential regions of *F8*. This proportion may vary according to the security of the initial haemophilia A diagnosis and the sensitivity of the mutation detection protocol in use. A proportion of these cases are likely to have mutations located in regions not routinely screened, such as deep within introns, and may only be detectable at the RNA level (Bagnall et al 1999) or be mediated by mutation mechanisms not yet elucidated (El-Maarri et al 2005). DNA sequencing may miss some mutation events, e.g. sequence duplications (Acquila et al 2004, Rost et al 2008) and heterozygous large deletions where a female sample is used for initial mutation screening. See also section 11 – Misdiagnosis.

5.5.1 1 stage - 2 stage assay discrepancy
Patients with mild haemophilia A may have a discrepancy in their FVIII level when measured with a one-stage assay (FVIII:C1) compared with a two-stage (FVIII:C2) or chromogenic assay. This discrepancy usually results in the one-stage level being higher than the two-stage level although an inverse discrepancy has also been described (Mumford et al 2002, see HAMSTeRS database for further examples of both types of discrepancy). Given that most laboratories in the UK currently perform the one-stage assay there is a possibility that some mild haemophilia A phenotypes may go undetected by standard coagulation analysis.

It is recommended that where mutations known to be associated with an assay discrepancy are detected, that this is highlighted in the mutation report.

5.5.2 Specific mutations association with inhibitor risk

Inhibitors (FVIII antibodies) are reported in up to one third of haemophilia A patients. Several factors contribute to the risk of inhibitor formation and *F8* mutation type is thought to contribute about 40% of this risk. Overall, large deletions show the highest risk, whilst missense mutations and splicing defects show the lowest risk (Schwaab et al 1995, Oldenburg et al 2002, Goodeve 2003, Coppola et al 2010).

However, an important consideration when reporting mutations associated with inhibitor formation is whether or not knowledge of this increased risk may influence treatment strategy when using replacement therapies. The UKHCDO Genetic Testing Network and Genetic Working Party discussed the inclusion of inhibitor risk in genetic analysis reports and the following consensus was achieved:

Severe haemophilia A

No comments relating to risk of inhibitor development as treatment strategy is unlikely to be affected.

Mild/moderate haemophilia A

Lab reports should indicate that a haemophilia A patient is at an increased risk of inhibitor formation if they have mild/moderate haemophilia A due to a p.Arg612Cys, p.Arg2169His or p.Trp2248Cys missense mutation (legacy nomenclature amino acid numbering is 593, 2150 and 2229 respectively). Data from HADB (accessed April 2010) showed that of patients with inhibitor data on the database, 15% of 41 patients with p.Arg612Cys had developed an inhibitor, 32% of 41 patients with p.Arg2169His and 38% of 13 patients with p.Trp2248Cys. This was in contrast to the remaining 4% of 689 patients with mild/moderate haemophilia A and a missense mutation who had developed an inhibitor.

It should be highlighted that this risk is experienced when a patient previously exposed only to DDAVP receives relatively large doses of replacement therapy to cover surgery etc. The following wording is suggested:

"The mutation p.aa1234aa has been shown to result in an increased risk of developing FVIII inhibitory antibody. Patients are at particular risk when they are exposed to replacement therapy to cover surgery etc."

5.6 Linkage analysis

Historically, linkage analysis was the method most commonly used to determine female carrier status in families with haemophilia A. Linkage studies are no longer in routine use within the UK as they have been superseded by direct mutation detection protocols (see section 5.2.4).

It is recommended that, wherever possible, mutation detection should be used for genetic counselling in haemophilia A families.

However, this is not always practicable, as the causative mutation cannot be identified in all families. Where direct mutation detection is not feasible, linkage analysis provides an acceptable alternative which offers a high degree of diagnostic confidence.

Intragenic linked markers 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 *F8*. 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.

It is recommended that where a mutation has not been found and linked markers are used, the diagnostic report should state that the possibility of non linkage of the phenotype to F8 cannot be excluded.

5.6.1 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.

For detailed information on intragenic markers applicable to haemophilia A carrier detection see the earlier published version of these guidelines (Keeney et al 2005).

Where demonstration of linkage using intragenic markers fails, the use of mutation screening is indicated.

6.0 PRENATAL DIAGNOSIS

Prenatal diagnosis (PND) is generally performed by chorionic villus sampling (CVS) 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 are in common usage. Female foetuses sexed by this method require confirmation that no maternal contamination is present in the sample. Both sexing and maternal contamination checks can be achieved by PCR amplification using commercially available kits that amplify, for example, multiple autosomal STRs to demonstrate the absence of maternal material in the foetal sample and also include sexing primers. A CMGS Practice Guideline on determination of maternal contamination is available (section 14). 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 working 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).

6.1 Non-invasive prenatal testing
Non-invasive prenatal testing has been applied to haemophilia A. Foetal gender can be determined by ultrasound analysis which can be corroborated by analysis of either cell-free foetal DNA or intact foetal cells from the maternal circulation. Foetal sex has been determined in blood samples of haemophilia carriers using amelogenin, *DYS14* and *SRY* genetic analysis (Santacroce et al 2006, Chi et al 2006). In an alternative approach, intact foetal cells are purified and can be used to seek a *F8* mutation in a male foetus (Sekizawa et al 2007). These approaches are not yet well established in a routine diagnostic setting in the UK.

6.1.1 Pre-implantation genetic diagnosis (PGD)
A proportion of women at risk of having a child with haemophilia do not wish to undergo invasive prenatal diagnosis and possible termination of pregnancy, or they may be undergoing fertility treatment. Pre-implantation genetic diagnosis (PGD) may be an option in these cases (Renwick and Ogilvie 2007, Lavery 2009).

6.1.2 Pre-implantation genetic haplotyping (PGH)
This is an alternative method for PGD enabling the selection of unaffected embryos in monogenic diseases in general. In PGH, a single blastomere is used for whole genome amplification (Renwick et al 2007, Laurie et al 2010). A panel of polymorphic markers surrounding the gene of interest are amplified and embryos screened for the affected allele(s) for which linkage has already been established in the family. Female embryos and males unaffected by haemophilia can then be implanted.
These non-invasive approaches are not yet well established in a routine diagnostic setting.

6.2 Late amniocentesis sampling
For women known to be haemophilia carriers who are carrying a male baby, but do not wish to pursue PND with a view to termination, options exist to inform clinical management of delivery. The haemophilia status of the male foetus can be determined by analysis of an amniotic sample taken late in pregnancy. This procedure should not be performed earlier than 35 weeks gestation in case it induces premature labour. Male foetuses shown not to carry the familial mutation can then be delivered in a setting of the mother's choice.

7.0 WORDING OF REPORTS

Reports must be clear, concise, accurate, fully interpretive, credible and authoritative. European guidelines which include laboratory testing and reporting recommendations are available (section 14 - EuroGentest reporting guidelines).

7.1 Mutation analysis reporting
Wording will depend on the confidence placed in the interpretation of any candidate mutation, as discussed in section 5.5. Suggested wording for a mutation which has a high confidence attached to it may include:

Mutation analysis in males
"x has a mutation (c.No.nt>nt, p.aaNo.aa), previously reported in F8 / 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 species, and/or is structurally or functionally important; this base change has been excluded as a common polymorphism by analysis of a panel of normal alleles; etc. Refer to section 5.5 for guidance.

Mutation analysis in females
"y carries a F8 mutation (c.No.nt>nt, p.aaNo.aa) which is consistent with carriership for the severity of haemophilia A seen in male relative x.

7.2 Linkage analysis using intragenic markers
The following wording is recommended:
The female can be diagnosed as a carrier/excluded from being a carrier, with a risk of error due to meiotic recombination of <1%.

8.0 NOMENCLATURE AND MUTATION REPORTING

8.1 Mutation nomenclature in use
Mutation reporting should ensure that the location of a given sequence change can be identified between laboratories. For guidance on nomenclature conventions used when reporting *F8* mutations refer to the recommendations of the Human Genome Variation Society (HGVS – section 14). Use of the HGVS system allows systematic identification of sequence alterations. In the case of *F8* the primary RefSeq in use (table 2) is the cDNA (RNA) sequence.

1. Use cDNA nucleotide numbering; +1 is the first base ("A") of the initiator methionine codon.

2. Amino acid numbering starts at +1 for the initiator methionine. Traditionally the system in use for FVIII amino acid numbering has started from the beginning of the mature protein sequence (see *Legacy Nomenclature* section below).

It is recommended that, for consistency with other genes, and in observance with HGVS guidance, it should be standard practice on diagnostic reports to number FVIII amino acids from the first methionine.

See section 8.2 for guidance on inclusion of legacy nomenclature in current reports.

3. Three letter amino acid codes should be used in the report to avoid confusion between amino acids and DNA bases.

4. The intron or exon containing the mutation should be stated.

5. A genomic reference sequence is available for *F8* (table 1). However, when reporting intronic changes, intronic numbering should be based on the cDNA nucleotide number of the closest end of the adjacent exon and: *"-" 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.*

6. For promoter nucleotides situated 5' of the initiator A, numbers are negative e.g. c.-123C>T

8.2 Legacy nomenclature

Note that databases such as HADB still give the legacy nomenclature convention for amino acid numbering of FVIII (accessed July 2010).

To convert from HGVS convention to this legacy amino acid numbering system which refers to the mature protein (Vehar et al 1984), subtract 19 from the HGVS amino acid number.

Given this legacy nomenclature it is essential practice to ensure that when reporting a mutation the nomenclature system in use is clearly stated.

It is recommended that the legacy numbering system should no longer be used in the main report result and interpretation sections but that it should be included on the report (possibly in a footnote or table legend) to enable comparison with previous family reports and the literature for mutations that have been previously reported. Where a family member has been previously reported using legacy nomenclature, both legacy and HGVS should be referred to in subsequent reports on the family (e.g. p.Tyr365Cys, previous nomenclature Y346C).

9.0 DETECTION OF LARGE SCALE DELETIONS IN HETEROZYGOUS CARRIERS

Large deletions involving *F8* occur in an estimated 2-5% of severe HA patients (Bogdanova et al 2005, Santacroce et al 2008) and HADB lists examples of larger deletions ranging from single exons up to the entire gene. Complex events involving both deletion and insertion have also been reported (Sheen et al 2007).

Large deletion mutations are readily detected in affected males due to lack of amplification of missing regions of *F8*. It is particularly difficult to detect these mutations in female relatives however, where the failure to amplify one of the two *F8* alleles must be detected. Possible methods for detection of female carrier status in these families include;

A multiplex ligation-dependent probe amplification (MLPA®) assay has been developed for *F8* (MRC Holland – section 14) and has been used to detect heterozygous deletions (Lannoy et al 2009, Santacroce et al 2009). Care should be taken in the use and interpretation of MLPA data. An Excel template to aid in data interpretation can be obtained from the Manchester National Genetic Reference Laboratory web site (section 14).

Where a female is being independently analysed in the absence of affected male relatives, laboratories may wish to add dosage analysis as a final stage in seeking the causative *F8* mutation.

- Gap PCR protocols may be developed where deletion boundaries are known
- Alternative methods based on gene dosage analysis may be utilised.
- Use of linkage analysis, which may reveal loss of heterozygosity for markers in the deleted region.

Neither gap PCR nor alternative dosage 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 apparent *de novo* change 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 should be included in the diagnostic report.

11.0 MISDIAGNOSIS

11.1 von Willebrand Factor – factor VIII binding analysis

A number of individuals with von Willebrand disease (VWD) have been previously misclassified as having mild haemophilia A or as haemophilia A carriers. 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. Type 2N VWD is an autosomal recessive inherited disorder. The two disorders can be discriminated by an ELISA based assay (VWF:FVIIIB), which determines the FVIII binding capacity of patient's VWF (Schneppenheim et al 1996, Caron et al 2002, Zhukov et al 2009). Some laboratories use this assay to examine all mild haemophilia A and VWD patients prior to their genetic analysis. Alternatively, the assay may be used where the FVIII deficiency does not show clear X-linked inheritance. Genetic analysis of the FVIII binding region of VWF can be undertaken to seek 2N VWD missense mutations (Keeney et al 2008).

11.2 Combined factor V and VIII deficiency

This rare condition may be misinterpreted as mild haemophilia A (Zhang and Ginsburg 2004). Patients typically have FVIII:C levels of 5-30 IU/dL resulting from autosomal recessive inheritance of mutations in the *LMAN1* or *MCFD2* genes (Zhang et al 2006). A non X-linked inheritance pattern, if apparent, and determination of both FVIII and FV levels in cases where a mutation is not found in *F8* should reveal the presence of this disorder.

11.3 Symptomatic female carriers

Carriers may be symptomatic (Plug et al 2006); approximately 10% of female haemophilia A carriers experience bleeding resulting from a reduced FVIII level. Females may be referred for analysis of the cause of their reduced VIII, even in the absence of any affected male relative. Their reduced FVIII could be due to carriership for haemophilia A with skewed X chromosome inactivation or possibly type 2N VWD. In cases where the family history provides no clue as to which disorder is present, the VWF-FVIII binding assay (VWF:FVIIIB) may be used, if available, to identify 2N VWD and/or sequence analysis of the VWF-FVIII binding region of the VWF gene (Keeney et al 2008).

12. EXTERNAL QUALITY ASSESSMENT & REFERENCE SAMPLES FOR TEST OPTIMISATION AND VALIDATION

An EQA scheme has been established for haemophilia A genetic investigation (Perry et al 2006). Details are available from UK NEQAS Coagulation, (section 14). The scheme examines clerical accuracy, correct identification of the nucleotide change according to HGVS guidance and the quality of report interpretation offered.

Participation in EQA is essential for UK laboratories performing haemophilia A genetic analysis.

The National Institute for Biological Standards and Control (NIBSC) have produced a World Health Organisation international standard gDNA reference material panel for the *F8* intron 22 inversion mutation, product code 08/160 (section 14).

13.0 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 (section 14).

13.1 Diagnostic turn around times

In line with the white paper publication "*Our inheritance, our future: realising the potential of genetics in the NHS"* (2003) the diagnostic turn around time (TAT) for non urgent investigations where the mutation is unknown is up to 8 weeks. For urgent samples, e.g. PND, the recommended TAT is 3 working days, assuming the mutation in the family is known.
For confirmation of a known mutation in a family the recommended TAT is 2 weeks although this is currently under review as it may not represent the best use of resources.

14.0 WEB RESOURCES

CMGS Best Practice Guidelines, including guidance on DNA sequencing and interpretation of unclassified variants, molecular analysis of VWD, maternal cell contamination: http://www.cmgs.org/BPGs/Best_Practice_Guidelines.htm

dbSNP, the NCBI Single Nucleotide Polymorphism database: http://www.ncbi.nlm.nih.gov/SNP/

EuroGentest Best Practice Guidelines for Molecular Genetic Testing:
http://www.acmg.net/Pages/ACMG_Activities/stds-2002/g.htm

Haemophilia Alliance, this is a UK wide partnership between patients with inherited bleeding disorders and health care professionals involved in the delivery of their care. The website includes the expected national service specification for haemophilia and other inherited bleeding disorders
http://www.haemophiliaalliance.org.uk/

HAMSTeRS (HADB), the Haemophilia A Mutation, Structure, Test and Resource Site has mutation and polymorphism databases for *F8* in addition to a partial molecular model of FVIII, methods pages and useful links to other sites: http://hadb.org.uk/

HGMD, the Human Gene Mutation Database. This collates mutation data on many disease genes. Access to the most up to date data requires a subscription:
http://www.hgmd.cf.ac.uk/ac/index.php

HGVS, the Human Genome Variation Society guidance on mutation nomenclature:
http://www.genomic.unimelb.edu.au/mdi/mutnomen/

MRC Holland, MLPA information and kits:
http://www.mrc-holland.com/WebForms/WebFormMain.aspx

NCBI RefSeq database, reference sequence resources. This contains current and archive versions of reference sequences given in table 2: http://www.ncbi.nlm.nih.gov/RefSeq/

NCBI Gene Review on haemophilia A, hyperlinked web based review on haemophilia A and its genetics: http://www.ncbi.nlm.nih.gov/bookshelf/br.fcgi?book=gene&part=hemo-a

NIBSC, the National Institute for Biological Standards and Control, produces international standard reference materials:
http://www.nibsc.ac.uk/

NGRL Manchester UK genetic service network support. Includes MLPA data interpretation aids and primer validation tools (SNPCheck): http://www.ngrl.org.uk/Manchester/index.html

Staden package for comparative sequence analysis:
http://staden.sourceforge.net/

UKHCDO Directory of Molecular Diagnostic Services for Inherited Bleeding Disorders: http://www.ukhcdo.org/.

UK Haemophilia Society. Represents those affected by bleeding disorders in the UK and lists all UK Haemophilia Centres, with Directors names and addresses:
http://www.haemophilia.org.uk/

UK NEQAS, National External Quality Assessment Service on Blood Coagulation, operates external quality assessment surveys on inherited bleeding disorder genetics:
www.ukneqasbc.org

World Federation for Hemophilia. Contains information for patients and healthcare professionals:
http://www.wfh.org/index.asp?lang=EN

16.0 REFERENCES

Acquila M et al. Duplication of exon 13 causes one third of the cases of mild hemophilia A in northern Italy. *Haematologica* 2004; 89: 758-759.

Antonarakis SS et al. Factor VIII gene inversions in severe haemophilia A-results of an international consortium study. *Blood* 1995; 86: 2206-2212.

Bagnall RD et al. Int22h-related inversions causing hemophilia A: a novel insight into their origin and a new more discriminant PCR test for their detection. *J Thromb Haemost* 2006; 4: 591-598.

Bagnall RD et al. Recurrent inversion breaking intron 1 of the factor VIII gene is a frequent cause of severe hemophilia A. *Blood* 2002; 99: 168-174.

Bagnall RD et al. Creation of a novel donor splice site in intron 1 of the factor VIII gene leads to activation of a 191 bp cryptic exon in two haemophilia A patients. *Br J Haematol* 1999; 107: 766-771.

Bogdanova N et al. Spectrum of molecular defects and mutation detection rate in patients with severe hemophilia A. *Hum Mutat* 2005; 26: 249-254.

Bogdanova N et al. Spectrum of molecular defects and mutation detection rate in patients with mild and moderate hemophilia A. *Hum Mutat* 2007; 28, 54-60.

Bolton-Maggs PHB, Pasi JK. Haemophilias A and B. *Lancet* 2003: 361; 1801-1809.

Bowen DJ, Keeney S. Unleashing the long-distance PCR for detection of the intron 22 inversion of the factor VIII gene in severe haemophilia A. *Thromb Haemost* 2003: 89; 201-202.

Caron C et al. Large experience with a factor VIII binding assay of plasma von Willebrand factor using commercial reagents. *Br J Haematol* 2002; 117: 716-718.

Chi C et al. Non-invasive first trimester determination of fetal gender: a new approach for prenatal diagnosis of haemophilia. *BJOG* 2006; 113: 239-242.

Cumming AM, on behalf of the UKHCDO Haemophilia Genetics Laboratory Network. The factor VIII intron 1 inversion mutation: prevalence in the UK. *J Thromb Haemost* 2004; 8: 205-206.

Coppola A et al. Understanding inhibitor development in haemophilia A: towards clinical prediction and prevention strategies. *Haemophilia* 2010; 16 Suppl 1:13-9.

Ellard S et al. Semi-automated unidirectional sequence analysis for mutation detection in a clinical diagnostic setting. *Genet Test Mol Biomarkers* 2009; 13: 381-386.

El-Maarri O et al. Analysis of mRNA in hemophilia A patients with undetectable mutations reveals normal splicing in the factor VIII gene. *J Thromb Haemost* 2005; 3: 332-339.

Forbes CD. The early history of hemophilia. In: Forbes CD, Aledort L, Madhok R, eds. *Hemophilia* 1997; Chapman & Hall, London, 3-6

Goodeve A. The incidence of inhibitor development according to specific mutations - and treatment? *Blood Coagul Fibrinolysis* 2003; 14 Suppl 1: S17-21.

Goodeve A. Molecular genetic testing of hemophilia A. *Semin Thromb Hemost* 2008; 34: 491-501.

Jayandharan G et al. Identification of factor VIII gene mutations in 101 patients with haemophilia A: mutation analysis by inversion screening and multiplex PCR and CSGE and molecular modelling of 10 novel missense substitutions. *Haemophilia* 2005; 11: 481-491.

Kasper CK, Lin JC. Prevalence of sporadic and familial haemophilia. *Haemophilia* 2007; 13: 90-92.

Kemball-Cook G et al. The Factor VIII Structure and Mutation Resource Site: HAMSTeRS Version 4. *Nucleic Acids Res* 1998; 26: 216-219.

Keeney S et al. UK Haemophilia Centre Doctors' Organisation Haemophilia Genetics Laboratory Network. The molecular analysis of haemophilia A: a guideline from the UK haemophilia centre doctors' organization haemophilia genetics laboratory network. *Haemophilia* 2005; 4: 387-97.

Keeney S et al. UK Haemophilia Centre Doctors' Organisation (UKHCDO). The molecular analysis of von Willebrand disease: a guideline from the UK Haemophilia Centre Doctors' Organisation Haemophilia Genetics Laboratory Network. *Haemophilia* 2008; 14: 1099-111.

Klopp N et al. 11 hemophilia A patients without mutations in the factor VIII encoding gene. *Thromb Haemost* 2002; 88: 357-360.

Lakich D et al. Inversions disrupting the factor VIII gene as a common cause of severe haemophilia A. *Nat Genet* 1993; 5: 236-241.

Lannoy N, et al. Identification of de novo deletion in the factor VIII gene by MLPA technique in two girls with isolated factor VIII deficiency. *Haemophilia* 2009; 15: 797-801.

Laurie AD et al. Preimplantation genetic diagnosis for hemophilia A using indirect linkage analysis and direct genotyping approaches. *J Thromb Haemost* 2010; 8: 783-9.

Lavery S. Preimplantation genetic diagnosis of haemophilia. *Br J Haematol* 2009; 144: 303-307.

Leuer M et al. Somatic mosaicism in hemophilia A: a fairly common event. *Am J Hum Genet* 2001; 69: 75-87.

Lin SY et al. Mutation spectrum of 122 hemophilia A families from Taiwanese population by LD-PCR, DHPLC, multiplex PCR and evaluating the clinical application of HRM. *BMC Med Genet* 2008; 9:53.

Liu Q et al. Single tube polymerase chain reaction for rapid diagnosis of the inversion hotspot of mutation in hemophilia A. Blood 1998; 92: 1458-1459. Erratum in Blood 1999; 93: 2141.

Liu Q, Sommer SS. Subcycling-PCR for multiplex long distance amplification of regions with high and low GC content: application to the inversion hotspot in the FVIII gene. Biotechniques 1998; 25: 1022-1028.

Miller CH et al. Reproductive choices in hemophilic men and carriers. *Am J Med Genet* 1987; 26: 591-598.

Mumford AD et al. Tyr346>Cys substitution in the interdomain acidic region a1 of factor VIII in an individual with factor VIII:C assay discrepancy. *Br J Haematol* 2002; 118: 589-594.

Naylor J et al. Characteristic mRNA abnormality found in half the patients with severe haemophilia A is due to large DNA inversions. Hum Mol Genet 1993; 2: 1773-1778.

Oldenburg J et al. Evaluation of DHPLC in the analysis of hemophilia A. *J Biochem Biophys Methods* 2001; 47: 39-51.

Oldenburg J et al. Inhibitor development in correlation to factor VIII genotypes. *Haemophilia* 2002; 8 Suppl 2: 23-29

Perry DJ et al. The UK National External Quality Assessment Scheme (UK NEQAS) for molecular genetic testing in haemophilia. *Thromb Haemost* 2006; 96: 597-601.

Plug I et al. Bleeding in carriers of haemophilia. *Blood* 2006: 108: 52-56.

Pruthi RK. Hemophilia: a practical approach to genetic testing. *Mayo Clin Proc* 2005; 80: 1485-99.

Renwick P, Ogilvie CM. Preimplantation genetic diagnosis for monogenic diseases: overview and emerging issues. *Expert Rev Mol Diagn* 2007;7:33-43.

Rossetti LC et al. Genotyping the hemophilia inversion hotspot by use of inverse PCR. *Clin Chem* 2005; 51: 1154-1158.

Rossetti LC et al. Developing a new generation of tests for genotyping hemophilia causative rearrangements involving int22h and int1h hotspots in the factor 8 gene. *J Thromb Haemost* 2008; 6: 830-836.

Rost S et al. Detection of large duplications within the factor VIII gene by MLPA. *Thromb Haemost* 2008; 6: 1996-1999.

Santacroce R et al. Identification of fetal gender in maternal blood is a helpful tool in the prenatal diagnosis of haemophilia. *Haemophilia* 2006; 12: 417-422.

Santacroce R et al. Identification of 217 unreported mutations in the *F8* gene in a group of 1,410 unselected Italian patients with hemophilia A. *J Hum Genet* 2008; 53: 275-284.

Santacroce R et al. Detection of new deletions in a group of Italian patients with Hemophilia A by multiplex ligation-dependent probe amplification. *Genet Test Mol Biomarkers* 2009; 13: 573-576.

Schneppenheim R et al. Results of a screening for von Willebrand disease type 2N in patients with suspected haemophilia A or von Willebrand disease type 1. *Thromb Haemost* 1996; 76: 598-602.

Schneppenheim R et al. The problem of novel FVIII missense mutations for haemophilia A genetic counseling. *Hamostaseologie* 2009; 29: 158-60.

Schwaab R et al. Haemophilia A: mutation type determines risk of inhibitor formation. *Thromb Haemost* 1995; 74: 1402-1406.

Sekizawa A et al. Recent advances in non-invasive prenatal DNA diagnosis through analysis of maternal blood. *J Obstet Gynaecol Res* 2007; 33: 747-764.

Sheen CR et al. Double complex mutations involving *F8* and FUNDC2 caused by distinct break-induced replication. *Hum Mutat* 2007; 28: 1198-1206.

Vehar GA et al. Structure of human factor VIII. *Nature* 1984; 312: 337-342.

Viel KR et al. A sequence variation scan of the coagulation factor VIII (FVIII) structural gene and associations with plasma FVIII activity levels. *Blood* 2007; 109: 3713-3724.

Vinciguerra C et al. Three abnormal patterns for inversion breaking intron 1 of the FVIII gene. *J Thromb Haemost* 2003; 1 (Supplement 1): OC128.

Vinciguerra C et al. Characterisation of 96 mutations in 128 unrelated severe haemophilia A patients from France. Description of 62 novel mutations. *Thromb Haemost* 2006; 95: 593-599.

White GC 2nd et al. Definitions in hemophilia. Recommendation of the scientific subcommittee on factor VIII and factor IX of the scientific and standardization committee of the International Society on Thrombosis and Haemostasis. *Thromb Haemost* 2001; 85: 560.

Zhang B, Ginsburg D. Familial multiple coagulation factor deficiencies: new biologic insight from rare genetic bleeding disorders. *J Thromb Haemost* 2004; 2:1564-1572.

Zhang B et al. Combined deficiency of factor V and factor VIII is due to mutations in either LMAN1 or MCFD2. *Blood* 2006; 107: 1903-1907.

Zhukov O et al. Measurement of von Willebrand factor-FVIII binding activity in patients with suspected von Willebrand disease type 2N: application of an ELISA-based assay in a reference laboratory. *Haemophilia* 2009; 15: 788-796.