

## Practice Guidelines for the Molecular Diagnosis of Haemophilia B.

Guidelines prepared by Mike Mitchell, Steve Keeney 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 9<sup>th</sup> 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	<i>Factor IX</i>
HUGO nomenclature	F9
OMIM Number	306900
Gene Cards ID	F9
Ensembl Gene ID	ENSG00000101981
Chromosomal location	Xq27.1-q27.2
Medline MESH term	Haemophilia B, factor-IX
NCBI LocusLink	HsF9 (Locus ID 2158)

### 2.0 DESCRIPTION OF THE DISEASE.

Haemophilia B or Christmas Disease is a recessively inherited X-linked bleeding disorder which results from deficiency of procoagulant factor IX (FIX). Factor IX deficiency is characterised by prolonged oozing after injuries, tooth extractions, or surgery, renewed bleeding after initial bleeding has stopped, and delayed bleeding. Severely affected males suffer from spontaneous joint and muscle bleeds and easy bruising. The age of diagnosis and frequency of bleeding episodes are related to the factor IX clotting activity. Haemophilia severity is defined by FIX: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). It is less common than haemophilia A with a frequency of approximately 1 in 25,000 males worldwide.

### 3.0 COMMON REASONS FOR REFERRAL

New cases of haemophilia B may be referred as a result of prior family history of the disease. In such cases male cord blood may be tested at birth to determine FIX:C. However, approximately one third of cases have no prior history of haemophilia B, these are referred to as having sporadic

disease. In these cases the age of diagnosis and frequency of bleeding episodes are often related to the factor IX clotting activity. Patients with severe haemophilia B are usually diagnosed during the first year of life. Without treatment, they have an average of two to five spontaneous bleeding episodes each month. Patients with moderately severe haemophilia B seldom have spontaneous bleeding; however, they do have prolonged or delayed oozing after relatively minor trauma and are usually diagnosed before the age of five to six years. The frequency of bleeding episodes varies from once a month to once a year. Patients with mild haemophilia B do not have spontaneous bleeding; however, without treatment, abnormal bleeding occurs with surgery, tooth extraction, and major injuries. The frequency of bleeding may vary from once a year to once every ten years. Patients with mild haemophilia B are often not diagnosed until later in life. In any patient, bleeding episodes may be more frequent in childhood and adolescence than in adulthood. 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 analysis is required to reliably determine female carrier status because the majority of female carriers have normal plasma factor IX levels. Carrier females with factor IX clotting activity <30% are at risk for bleeding (approximately 10% of carrier females, independently of severity of disease in their family).

Genetic counselling should be performed by suitably qualified 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.

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).

### 4.0 THE GENE

The factor IX gene, located on the long arm of the X chromosome at Xq27, spans 33.5kb of DNA and comprises 8 exons.

FIX mRNA is 2.8kb and encodes a mature protein of 415 amino acids. Haemophilia B results from heterogeneous mutations spread throughout the FIX gene. Unlike haemophilia A, no common repeat mutation has been identified. However, 20-30% of cases of mild haemophilia B are due to a small number of founder mutations. Exon 8 is the largest FIX exon, being 1.9kb in length and

representing almost half of the FIX coding region. Half of all FIX mutations are found in this exon. Mutations in the promoter of the factor IX gene are relatively rare (~2% of the total) but important because they give rise to the unique Haemophilia B Leyden phenotype, where symptoms typically ameliorate at puberty from severe to mild or even asymptomatic. An annually updated international mutation database is maintained by Dr. Peter Green at Guy's Hospital, London (URL: [www.kcl.ac.uk/ip/petergreen/haemBdatabase.html](http://www.kcl.ac.uk/ip/petergreen/haemBdatabase.html)).

## 5.0 APPROACHES AND PROTOCOLS

### 5.1 Previously Characterised Mutations

The Division of Medical & Molecular Genetics at Guy's Hospital, London, has characterised the mutation(s) responsible for haemophilia B in a large proportion of UK families. However, this programme was never intended as a diagnostic service and is no longer active. Many laboratories are now screening their patients for mutations in the FIX gene (see the UKHCDO Directory of Molecular Diagnostic Services for Inherited Bleeding Disorders, hosted by the British Society for Haematology (BSH)). 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 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 B population rely on heteroduplex formation and subsequent detection of mismatched heteroduplexes. There are two major heteroduplex formation methods in current use; CSGE and dHPLC.

#### 5.2.1 CSGE

Conformation sensitive gel electrophoresis (CSGE, Ganguly et al, 1993) is a variant of heteroduplex analysis applied to the screening of factor IX gene mutations (Hinks et al 1999). 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) when amplifying large exons such as exon 8. Given careful design and application an expected mutation detection sensitivity of >90% can be expected.

#### 5.2.2 dHPLC

Denaturing high performance liquid chromatography (dHPLC, 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 FIX gene (Castaldo et al, 2003).

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 (e.g. Montandon et al 1989 & Tartary et al 1993) but are not currently employed by the network.

### 5.3 FIX Deletions

Patients having partial or complete factor IX gene deletions are relatively rare, comprising only ~3% of patients. Deletions may also be demonstrated by apparent non-inheritance of an allele within a family. The detection of large deletions is often difficult in heterozygous carriers. Currently methodologies measuring gene dosage e.g. Quantitative PCR/RT-PCR or loss of heterozygosity are the best option, although not infallible. Newer methodologies are available for exploration e.g. Multiplex Ligation-dependent Probe Amplification (MLPA).

### 5.4 Direct Sequencing

DNA sequencing is considered the gold standard for mutation detection. The FIX gene is small enough to contemplate sequencing the coding region, splice junctions, and the 5' & 3' regions for previously unknown mutations (Vidal et al 2000), within the time constraints of a diagnostic service. 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.

Once a mutation is identified in a family, direct sequencing is the preferred methodology with which to confirm or exclude its' presence in other family members.

**NB Direct sequencing is not infallible when detecting heterozygous base changes. 'Preferential incorporation' may lead to heterozygotes being missed. 'Heterozygote sequencing' should always be performed both 5' (Forward) and 3' (Reverse). Design of a 'mutation specific test' e.g. restriction digest or allele specific PCR is an alternative approach to consider.**

#### 5.4.1 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 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 in both 5' (forward) and 3' (reverse) directions.

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 sample and re-amplification 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.

### 5.5 Mutation Validation

When a novel nucleotide change is found, caution should be exercised before deciding that it is the one responsible for disease. Whereas termination, deletion and insertion mutations may obviously be causative, missense and other changes may not.

The Haemophilia B Database can be consulted to determine whether the change has been previously reported in a patient having similar disease severity.

#### **Minimum checks should include:**

- Does the reported severity agree with the levels in the patient being analysed?
- Has the candidate mutation been reported previously as a polymorphism?
- Is an amino acid change likely to be pathogenic e.g. is the change non-conservative in nature or is it within a recognized functionally important region of the protein?
- Could the candidate mutation affect splicing? Software tools, e.g. StrataSplice or the NetGene2 server can be used to allow alternative splice site prediction.
- Is the changed amino acid conserved across species?
- Could the ethnic origin of the patient affect interpretation of polymorphism/candidate mutation status for a given base change?

The family should be tested to determine whether the nucleotide alteration tracks with the disease and a panel of normal DNA samples of the same ethnic origin (where possible) examined to rule out a polymorphic change. Wherever possible candidate mutations should be confirmed in affected, or excluded in unaffected, males on the maternal side of the family. Remember that candidate mutations can still be used as bespoke genetic markers if they track appropriately within the family, irrespective of their disease association.

### 5.6 Linkage Analysis

Historically, linkage analysis was the method most commonly used to determine female carrier status in families with haemophilia B. Linkage studies have been superseded by direct mutation analysis protocols. However, intragenic linked markers may still be useful in 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

The use of intragenic markers only should be considered.

The entire ~35kb of the FIX gene has been sequenced, but no short tandem repeat polymorphisms have been found. However, the *DdeI* polymorphism in intron 1 is a complex

repeat (Figuredo et al, 1994) having two common and several rarer alleles and a Caucasian heterozygosity rate of 35%, making it a useful component of a panel of markers for linkage analysis.

There are eleven diallelic polymorphisms throughout the FIX gene which can also be used in linkage analysis. Their rate of heterozygosity varies quite markedly between different ethnic groups. In Caucasians, a combination of just three markers, *MseI* in the 5' untranslated region (Winship et al, 1996), *DdeI* (Bowen et al, 1991) and *HhaI* (Winship et al, 1989) in the 3' flank of FIX give a combined heterozygosity rate of ~80%. A combination of markers (Goodeve 1998) can achieve informativity in ~95% of Afro-Caribbean families, 85-90% of Caucasian families and ~60% of Asian families. As all of these polymorphisms lie within or very closely flank the FIX gene, the rate of recombination between any marker and the mutation is well under 1%.

FIX polymorphisms may be analysed very simply. Naturally occurring or introduced restriction enzyme sites are available for each polymorphism, so each marker can be PCR amplified, restriction enzyme digested, electrophoresed and visualised rapidly.

It should be noted that linkage analysis fails in a number of families for one of the following reasons;

- Lack of prior family history (although exclusion may still be possible).
- Key pedigree members not available.
- Polymorphisms uninformative in key female(s).
- Non-paternity.
- 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 B families. However, this may not always be practicable and, 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 (PND)

Prenatal diagnosis is generally performed by chorionic villus sampling at between 11 and 13 weeks of gestation. Direct karyotype analysis may 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. Male haemophilia status can be determined by analysis of a previously determined informative marker or familial mutation. As all of these analyses for FIX involve PCR amplification, results should be provided within 2-3 days of the CVS sample being taken.

Detailed discussion of issues of PND can be found in the UKHCDO document "Clinical Genetics Services for Haemophilia" (ISBN 901787 07 9).

**Table 2** Commonly analysed FIX polymorphisms

Primer designation	Sequence 5'-3'	PCR product size/bp*	Heterozygosity#	Reference
HhaI	(F) ACA GGC ACC TGC CAT CAC TT (R) AGA TTT CAA GCT ACC AAC AT	- 230bp + 150 & 80bp	0.48	Winship et al, 1989
DdeI	(F) GGG ACC ACT GTC GTA TAA TGT GG (R) CTG GAG GAT AGA TGT CTC TAT CTG	369bp large common allele 319bp small common allele	0.36	Bowen et al, 1991
MseI	(F) GAT AGA GAA ACT GGA AGT AGA CCC (R) TTA GGT CTT TCA CAG AGT AGA ATT T	undigested = 369bp (-) 176 & 83 & 73 & 37bp (+) 176 & 57 & 26 & 73 & 37 bp	0.44	Winship et al, 1993
"SalI"	(F) CTC GTT GTGCACATG TAC CC (R) CAA TAC CAC CCT ATC CTT CGT CGA	- 317bp + 295 & 22bp	0.49	Toyozumi et al, 1995
MnII	(F) AAG TGA CAA GGA TGG GCC TCA ATC (R) GAA ACT TGC CTA AAT ACT TCT C	undigested = 422bp (-) 333 & 62 & 27bp (+) 214 & 119 & 62 & 27bp	0.44	Tsang et al, 1989

Key: \* + Indicates presence of restriction site, - indicates absence; #Caucasian heterozygosity rate, see Goodeve (1998) or Goodeve & Peake (1997) for other ethnic groups

## 7.0 WORDING OF REPORTS

Reports must be clear, concise, accurate, fully interpretive, credible and authoritative. For general guidance on reporting 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 5.5. Suggested wording for a mutation which has a high confidence attached to it may include:

#### 7.2.1 Mutation analysis in males

*"x has a FIX mutation (No.nt>nt, aaNo.aa), previously reported in the FIX gene/not previously reported. The mutation is consistent with the severity of haemophilia B 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/functionally important; this base change has been excluded as a common polymorphism by analysis of >100 normal alleles; etc.

#### 7.2.2 Mutation analysis in females

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

### 7.3 Mutation 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.

Nucleotides and amino acids should be numbered as per Yoshitake et al (1985). Nucleotide numbering starts from 'the proposed transcription initiation site' +1 and is continuous through introns. Amino acid numbering starts at +1 for the first codon of the mature protein (tyrosine). The signal peptide is numbered from -46 (initiator methionine) to -1 (arginine). The intron or exon containing the mutation should be stated. Conventionally factor IX exons have been labelled A-H and the introns have been numbered 1-7. A more standard numbering system of 1 to 8 for exons may be used instead, if preferred. The use of lower case letters for intronic sequence is recommended. To avoid potential confusion between single letter amino acid codes and nucleotides the following convention is recommended: *Nucleotide position 17711G>A, corresponding amino acid position C99Y.* 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".

## 8.0 MOSAICISM

Germline and somatic mosaicism may complicate any genetic diagnosis in haemophilia B. 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 (although not exclusively) where the apparently *de novo* mutation is a point mutation.

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. Alternatively, a specific reference to the possibility of germline mosaicism may be added.

## 9.0 REFERENCE SAMPLES FOR TEST OPTIMISATION AND VALIDATION

Reference samples have yet to be established for haemophilia B.

## 10. LABORATORY MATERIALS

### 10.1 PCR Primer Sequences

See Section 5.6 above.

### 10.2 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, hosted by the BSH.

## 11. WEB RESOURCES

Haemophilia B database

(<http://www.kcl.ac.uk/ip/petergreen/haemBdatabase.html>)

Annually updated list of reported mutations in FIX.

UK Haemophilia Society

(<http://www.haemophilia.org.uk/>)

Lists all UK Haemophilia Centres, with Directors names and addresses.

World Federation for Hemophilia

(<http://www.wfh.org/index.asp?lang=EN>)

UKHCDO Genetic Network Directory

Directory of molecular diagnostic services for inherited bleeding disorders in the U.K. (<http://www.ukhcd.org/>).

Web site of the Clinical Molecular Genetics Society – includes Best Practice Guidelines.

(<http://cmgsweb.shared.hosting.zen.co.uk/>)

## 12.0 REFERENCES

### 12.1 General Information

White GC 2nd et al (2001). Factor VIII and Factor IX Subcommittee. 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.* 85: 560.

Giannelli F et al (1998). Haemophilia B: database of point mutations and short additions and deletions - eighth edition. *Nucl Acids Res* 26: 265-268.

Bowen DJ (2002). Haemophilia A and haemophilia B: molecular insights. *Mol. Pathol.* Apr 55: 127-44.

Bolton-Maggs PHB, Pasi JK (2003). Haemophilias A and B. *The Lancet*; 361: 1801-9.

Goodeve AC (1998). Laboratory methods for the genetic diagnosis of bleeding disorders. *Clin Lab Haematol*, 20: 3-19.

Goodeve AC, Peake IR (1997). Diagnosis of haemophilia A carriers and prenatal diagnosis. In: Forbes CD, Aledort L, Madhok R, eds. *Hemophilia*. Chapman & Hall, London, 63-74.

Peake IR et al (1993). Report of a joint WHO/WFH meeting on the control of haemophilia: carrier detection and prenatal diagnosis. *Blood Coag Fibrinol* 4: 313-344.

UKHCDO Genetic Guidelines (1997). UK Haemophilia Centre Directors Organisation, Oxford.

Yoshitake S et al (1985). Nucleotide sequence of the gene for human factor IX (antihemophilic factor B). *Biochemistry* 24: 3736-3750.

### 12.2 Point Mutation Analysis

Ganguly A et al (1993). Conformation sensitive gel electrophoresis for rapid detection of single base differences in double-stranded PCR products and DNA fragments: Evidence for solvent-induced bends in DNA heteroduplexes. *Proc Natl Acad Sci USA* 90: 10325-10329.

Hinks JL et al (1999). A rapid method for haemophilia B mutation detection using conformation sensitive gel electrophoresis. *Br J Haematol* 104: 915-8.

Montandon AJ et al (1989). Direct detection of point mutations by mismatch analysis: application to haemophilia B. *Nucl Acids Res* 17: 3347-3358.

Tartary M et al (1993). Detection of a molecular defect in 40 of 44 patients with haemophilia B by PCR and denaturing gradient gel electrophoresis. *Br J Haematol* 84: 662-669.

Oefner PJ, Underhill PA (1995). Comparative DNA sequence by denaturing high performance liquid chromatography (DHPLC). *Am J Hum Genet* 57:A266.

Oefner PJ, Underhill PA (1998). DNA mutation detection using denaturing high-performance liquid chromatography (DHPLC). *Curr Protocols Hum Genet* 19(Suppl): 7101-71012.

Castaldo G et al (2003). Denaturing HPLC procedure for factor IX gene scanning. *Clin. Chem.* 49: 815-818.

O'Donovan, M.C et al (1998). Blind analysis of denaturing high performance liquid chromatography as a tool for mutation detection. *Genomics* 52: 44-49.

Vidal F et al (2000) Factor IX gene sequencing by a simple and sensitive 15-hour procedure for haemophilia B diagnosis: identification of two novel mutations. *Br J Haematol*. 111:549-51.

### **12.3 Linkage Analysis**

Bowen DJ et al (1991). Facile and rapid analysis of three DNA polymorphisms within the human factor IX gene using the polymerase chain reaction. *Br J Haematol* 77: 559-560.

Figueiredo MS et al (1994). Factor IX gene haplotypes in Brazilian Blacks and characterization of unusual Dde I alleles. *Br J Haematol* 87: 789-796.

Tsang TC et al (1989). The use of DNA amplification for genetic counselling related diagnosis in haemophilia B. *Thromb Haemost* 61: 343-347.

Toyozumi H et al (1995). Diagnosis of haemophilia B carriers using two novel dinucleotide polymorphisms and Hha I RFLP of the factor IX gene in Japanese subjects. *Thromb Haemost* 74: 1009-1014.

Winship PR et al (1989). Detection of polymorphisms at cytosine phosphoguanadine dinucleotides and diagnosis of haemophilia B carriers. *Lancet* i, 631-634.

Winship PR et al (1993). An Mse I RFLP in the 5' flanking region of the factor IX gene: its use for haemophilia B carrier detection in Caucasian and Thai populations. *Br J Haematol* 84: 101-105.