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Assay Summary

Factor VIII Gene Mutation Analysis

Hemophilia A

Synopsis

Identification of causative mutations in the factor VIII gene (X chromosome) for families with hemophilia A can permit very accurate determination of carrier status of at-risk females in these families and provides options for prenatal diagnosis. To identify the mutation in a hemophilia A family, a blood sample is required from an affected individual (proband). Because inversions in intron 22 of the factor VIII gene are found in approximately 45% of patients diagnosed with severe hemophilia A, the intron 22 gene inversion analysis is initially performed on samples from patients with severe hemophilia A. For cases of mild or moderate disease or for those with severe disease and not having a gene inversion, comprehensive mutation analysis can be performed. This analysis entails full gene sequencing of the factor VIII gene and, if negative, further analyses for deletion/duplication, the factor VIII gene intron 1 inversion and von Willebrand Factor Normandy mutations. The intron 1 inversion is present in about 5% of patients with severe hemophilia A. Large deletions/duplications account for approximately 6% of hemophilia A gene alterations. The von Willebrand factor is a protein that complexes with and stabilizes factor VIII in plasma. Mutations in the factor VIII binding site of von Willebrand factor cause a phenotype similar to mild hemophilia A called von Willebrand disease, Type 2N (Normandy). Please also see our Assay Summary for von Willebrand factor Normandy.

After a mutation is detected in a proband, carrier testing and prenatal diagnosis may be offered to the family. In cases where a proband is not available for testing, analysis may be performed on a sample from an obligate carrier.

Indications for testing

Individuals with a diagnosis of hemophilia A, appropriate at-risk female relatives of probands with identified mutations, and hemophilia A carriers with previously identified factor VIII gene mutations desiring prenatal diagnosis, with genetic counseling, are candidates for testing.

Methodology

Factor VIII intron 22 inversion analysis: Factor VIII intron 22 inversions are detected by a novel DNA amplification assay developed in this laboratory¹. The method is specific for factor VIII gene inversions involving sequences in intron 22. The method will distinguish between patients with the inversion and female carriers of the inversion.

Factor VIII sequence analysis: All the coding regions and splice junctions of the factor VIII gene are analyzed by direct DNA sequence analysis using an automated fluorescent sequencing machine. When a mutation is detected, confirmation is carried out on an independent amplification of PCR using a second prep (B-prep) by sequencing in the opposite direction. If no mutation is found, sequence analysis is performed in both directions. At-risk family members can be offered DNA sequence analysis of only the region of the gene with the previously identified mutation.

Factor VIII deletion/duplication analysis: Factor VIII large deletions are found in about 6% of hemophilia A. Testing in females for large deletions and duplications or males for large duplications is performed by SALSA Multiplex Ligation-Dependent Probe Amplification (MLPA)⁴, a rapid, high-throughput technique for copy number quantification. This assay should be considered for patients with Hemophilia A where full gene sequencing and inversion testing did not detect a mutation. The probe mixes included in this MLPA kit contain probes for all 26 exons of F8 gene. More than one probe is present for exons 1, 7, 12, 14 and 26. This probemix does not contain a probe for exon 22B which is only present in an alternative transcript.

Factor VIII intron 1 inversion analysis: Factor VIII intron 1 inversions are detected using two complementary amplification methods to detect rearrangement of factor VIII intron 1 sequences^{2, 3}.

Von Willibrand Factor Normandy sequence analysis: For detection of von Willebrand factor Normandy mutations, three exons (18, 19, and 20) of the von Willebrand factor gene are analyzed by direct DNA sequence analysis using an automated fluorescent sequencing machine. When a mutation is detected, confirmation is carried out on an independent amplification of PCR using a second prep (B-prep) by sequencing in the opposite direction. If no mutation is found, sequence analysis is performed in both directions. At-risk family members can be offered DNA sequence analysis of only the region of the gene with the previously identified mutation.

Performance

Intron 22 and intron 1 Inversion Analysis: Compared to Southern blot analysis, the analytical sensitivity for detection of factor VIII gene inversions in hemophilia A patients is greater than 99%; the specificity is greater than 97%. The sensitivity and specificity for carrier detection and for prenatal diagnosis for families with identified factor VIII gene inversions are both estimated to be greater than 99%.

Factor VIII full gene sequencing: Direct sequencing is very sensitive, but multiple factors, including genetic heterogeneity and mutations outside of the regions of likely functional significance, imply that mutations will not be detected in about 2-5% of families. Thus, the sensitivity for mutation detection in hemophilia A patients is approximately 95-98%. Once a mutation is found, the sensitivity and specificity for carrier detection and for prenatal diagnosis for families with identified factor VIII gene mutations are both estimated to be greater than 99%.

MLPA analysis of the factor VIII gene has demonstrated very good analytical sensitivity. Validation of the assay in our lab was able to detect multiple exons deletions and duplications in all cases, with 100% concordance with another large deletion/duplication analysis methodology (Robust Dosage-PCR). This gives an estimated analytical sensitivity of greater than 98%.

Limitations

Factor VIII intron 1 & 22 inversion analysis: Inversion analysis will only detect factor VIII gene inversions involving introns 22 and 1 sequences. Mutation analysis will not detect mutations outside of the regions analyzed or any inversion. Thus, inversion analysis should be performed prior to full mutation analysis for all cases of severe hemophilia A.

Factor VIII & vWF Normandy sequence analysis: The sequence analysis will not detect mutations located in regions of the Factor VIII and von Willebrand Factor (Normandy) genes that are not analyzed (non-coding exon regions, intron regions other than the splice junctions, and upstream and downstream regions). The sequencing also will not detect gross genetic alterations including most duplications, inversions, or deletions (in females for Factor VIII). Some sequence alterations that may be detected (such as those causing missense or synonymous changes) will be of unknown clinical significance.

MLPA analysis for large deletions/duplications: The MLPA analysis will not detect mutations located in regions of the Factor VIII gene that are not analyzed (excluded exons, introns, and upstream or downstream regions not analyzed). MLPA analysis will not detect sequence alterations or inversions. Furthermore, mutations and/or polymorphisms very close to the probe ligation site may also result in a

reduced relative peak area. Therefore, apparent deletions detected by a single probe always require confirmation by other methods.

Interpretation of test results should be in the context of the patient's ethnicity, clinical and family histories, and other laboratory test results.

Note: Prenatal diagnosis is available for a male fetus once positive carrier status has been established for the mother.

Specimen Requirements

(a) Blood samples: 2 tubes with a total of 6 ccs in ACD (yellow top) or EDTA (lavender top) tubes. Keep at ambient temperature and ship by overnight courier. Samples must be received in our laboratory within 72 hours of draw.

Note:

- i) for infants, a minimum of 3 ccs is sufficient.
- ii) we accept DNA; at least 10 micrograms is required.

(b) Prenatal samples: 2 T25 flasks of confluent cells sent padded to arrive on M/Tu/W.

A blood sample from the mother maybe required (2 tubes with a total of 6 ccs in ACD (yellow top) or EDTA (lavender top) tubes) for use as positive control. Maternal cell contamination studies are not done here but are required for autosomal disorders and dosage analysis on X-linked disorders. We would be happy to assist in coordinating sending out a specimen for this purpose.

Test Request Form (TRF)

- (a) A completed MDL [TRF](#) is required for each specimen. Please submit the completed TRF with the specimen. Complete testing and billing information must be provided before the specimen is processed.
- (b) [Hemophilia Patient Information Form](#): Include a completed Hemophilia Patient Information Form for the proband and a complete pedigree.

<i>Order Codes</i>	<i>CPT Codes</i>	<i>TAT</i>
F8-SEQ (Factor VIII gene, full gene sequencing)	83890, 83898(x30), 83904(x35), 83894, 83912	8 wks
F8-CAS (Factor VIII gene, known mutation detection, carrier)	83890, 83898, 83904, 83894, 83912	3 wks
F8PBIa (Factor VIII gene, intron 22 inversion analysis)	83890, 83900(x2), 83901, 83894, 83912	3 wks
F8INV1 (Factor VIII gene, intron 1 inversion analysis)	83890, 83900(x2), 83901, 83894, 83912	3 wks
F8-INV22-CAS (Factor VIII gene, intron 22 inversion analysis, carrier)	83890, 83900(x2), 83901, 83894, 83912	3 wks
F8-INV1-CAS (Factor VIII gene, intron 1 inversion analysis, carrier)	83890, 83900(x2), 83901, 83894, 83912	3 wks
F8PDIa (Factor VIII gene, intron 22 inversion analysis, prenatal)	83890, 83900(x2), 83901, 83894, 83912	2 wks
F8-INV1-PD (Factor VIII gene, intron 1 inversion analysis, prenatal)	83890, 83900(x2), 83901, 83894, 83912	2 wks
F8PDS (Factor VIII gene, known mutation detection, prenatal)	83890, 83898, 83904, 83894, 83912	2 wks
F8-DEL (Factor VIII gene, MLPA analysis, 26 Exons)	83890, 83896(x33), 83909, 83912	3 wks
F8-DEL-CAS (Factor VIII gene, MLPA analysis, known deletions/duplications)	83890, 83896(x33), 83909, 83912	3 wks
F8-DEL-PD (Factor VIII gene, MLPA analysis, prenatal)	83890, 83896(x33), 83909, 83912	3 wks
VWFNa (von Willebrand Factor, Normandy, full gene sequencing)	83890, 83898(x6), 83904(x3), 83894, 83912	4 wks

References

1. Liu, Q. et al. (1998). Blood 92:1458-1459.
2. Brinke, A. et al. (1996) Hum Mol Genet 5:1945-1951.
3. Bagnall, R.D. et al. (2002) Blood 99:168-174.
4. Schouten JP et al. (2002) *Nucleic Acids Res* 30, e57

NOTE: This test is performed pursuant to a license agreement with Roche Molecular Systems, Inc.