Chapter 3 **LABORATORY DIAGNOSIS AND MONITORING**

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RECOMMENDATIONS

3.1 | Introduction

Recommendation 3.1.1

The WFH recommends that testing for diagnosis and monitoring of hemophilia must be carried out by staff with knowledge and experience in coagulation laboratory testing using equipment and reagents that have been validated for this specific purpose.

• REMARK: Details of laboratory tests for the diagnosis and monitoring of hemophilia are described in the WFH laboratory manual. **CB**

3.2 | Coagulation laboratory testing

Recommendation 3.2.1

In preparation for collection of a blood sample for determination of prothrombin time (PT), activated partial thromboplastin time (APTT), or FVIII/FIX activity, the WFH advises that patients with hemophilia may maintain their regular diet—overnight fasting is not necessary prior to blood draw.

• REMARK: High levels of lipid in the plasma may affect the determination of clotting times when using coagulometers with optical systems. **CB**

Recommendation 3.2.2:

In preparation for collection of a blood sample for determination of APTT or FVIII/FIX activity, the WFH recommends that patients with hemophilia avoid strenuous exercise prior to blood draw.

• REMARK: Strenuous exercise or stress can temporarily elevate FVIII activity of patients with mild hemophilia A into the reference range; therefore, patients should be rested for a few minutes prior to venipuncture. **CB**

Recommendation 3.2.3:

For the diagnosis and monitoring of hemophilia A and B, the WFH recommends that blood samples be labelled immediately with the patient ' s first and last name, an identification number or date of birth, and the date and time of specimen collection. This should be done before leaving the side of the patient.

• REMARK: There is no consensus on whether the tube should be labeled immediately before or immediately after blood collection. **CB**

Recommendation 3.2.4:

The WFH recommends that blood samples for determination of PT, APTT, or FVIII/FIX activity be collected in citrate tubes containing 0.105-0.109M (around 3.2%) aqueous trisodium citrate dihydrate, capped during processing, and kept at 18-25°C during transport and storage. Blood samples should be centrifuged at ambient temperature for a minimum of 1700 g for at least 10 minutes, and either be analyzed within 8 hours of collection (4 hours for FVIII:C) or stored deep frozen at -35°C or lower.

• REMARK: Storage of citrated whole blood samples at 2-8°C should be avoided as this may result in loss of FVIII activity.

• REMARK: Platelet poor plasma (PPP) samples can be stored at -35°C for up to 3 months and at -70°C for up to 6 months prior to determination of FVIII/FIX activity. Storage of PPP at -20°C is usually inadequate. Freezers with auto-defrost should not be used to store PPP prior to determination of PT, APTT, or FVIII/ FIX activity. **CB**

Recommendation 3.2.5:

The WFH recommends that blood samples for determination of PT, APTT, or FVIII/FIX activity should be rejected and replaced if the collection tube contains less than 80% of the target fill volume.

• REMARK: If the collection tube contains between 80% and 90% of its target fill volume, the results obtained using certain methods may have minor artefactual prolongation of PT and APTT and minor artefactual reduction in FVIII/FIX activity. **CB**

Recommendation 3.2.6:

The WFH recommends that blood samples for determination of APTT or FVIII/FIX activity should be rejected and replaced if in vitro hemolysis or clotting have occurred during the collection and processing of the sample.

• REMARK: The impact of in vitro hemolysis on PT is insufficient to affect patient management.

• REMARK: Samples from patients with in vivo hemolysis that have been collected for determination of PT, APTT, or FVIII/FIX activity can be accepted and tested. **CB**

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Recommendation 3.2.7:

For laboratory investigation of patients being assessed due to clinical suspicion of hemophilia A, the WFH recommends that prothrombin time testing also be performed using a laboratory reagent containing human tissue factor.

• REMARK: Hemophilia A is sometimes excluded despite clinical suspicion of its presence. Such cases may have other factor deficiencies. Some patients with certain FVII defects may have symptoms similar to mild hemophilia but may display normal PT and FVII activity if the laboratory reagent contains non-human tissue factor so that the diagnosis would be missed. **CB**

Recommendation 3.2.8:

For laboratory investigation of patients being assessed due to clinical suspicion of hemophilia, the WFH recommends that an APTT result within the reference range not be used to rule out the presence of mild hemophilia A or B.

• REMARK: In some cases of mild hemophilia A or B, APTT may be within the normal range. **CB**

Recommendation 3.2.9:

The WFH recommends that an APTT result within the normal range obtained in a sample containing an equal volume mixture of patient and pooled normal plasma that was analyzed immediately after preparation of that mixture should not be used to rule out the possible presence of an FVIII inhibitor.

• REMARK: The APTT of an equal volume mixture of patient and pooled normal plasma becomes substantially prolonged over a period of 1 to 2 hours of incubation at 37°C if the patient sample contains a neutralizing anti-FVIII inhibitor. **CB**

Recommendation 3.2.10:

For laboratory investigation of patients being assessed due to clinical suspicion of hemophilia A, the WFH recommends the use of both the one-stage FVIII assay and the chromogenic FVIII:C assay in the initial diagnostic workup.

• REMARK: Both assays should be performed even if the result of one of the two assays shows FVIII activity within the normal range.

• REMARK: The one-stage FVIII assay requires the use of FVIII deficient plasma containing less than 1 IU/dL (<1%) FVIII activity and normal levels of other clotting factors that can influence APTT (fibrinogen, FII, FV, FIX, FX, FXI, FXII, prekallikrein, and HMWK). **CB**

Recommendation 3.2.11:

For laboratory investigation of patients being assessed due to clinical suspicion of hemophilia B, the WFH recommends the use of the one-stage FIX assay in the initial diagnostic workup.

• REMARK: Data are currently insufficient to make recommendations on the role of the chromogenic FIX assay in the initial diagnostic workup of hemophilia B.

• REMARK: The one-stage FIX assay requires the use of FIX deficient plasma containing less than 1 IU/dL (<1%) FIX activity and normal levels of other clotting factors that can influence APTT (fibrinogen, FII, FV, FVIII, FX, FXI, FXII, prekallikrein, and HMWK). **CB**

Recommendation 3.2.12:

For one-stage or chromogenic FVIII/FIX assays, the reference/standard plasma used for calibration, whether commercially or locally prepared, must be traceable to a WHO international standard, and results should be reported in international units (IUs).

• REMARK: Results should be reported as IU/mL or IU/dL.

• REMARK: In principle, percentage is the appropriate unit of activity only when the assay is performed using pooled normal plasma as the reference plasma whose activity is not traceable back to a WHO international standard. **CB**

Recommendation 3.2.13:

For laboratory investigation due to clinical suspicion of hemophilia using one-stage FVIII/FIX assays, the WFH recommends analysis using 3 different dilutions of test plasma samples.

• REMARK: The results of the test and standard plasma dilutions should be compared by parallel-line analysis. One way to assess this is to calculate the coefficient of variation (CV) of the 3 results using the equation CV = ([standard deviation/ mean] × 100). If the CV of the 3 results is less than 15%, then the average of the 3 results should be reported. If the CV is greater than 15%, the results should be scrutinized. Presence of pathological inhibitors against specific clotting factors or lupus anticoagulants can interfere with some one-stage FVIII and FIX assays. Some therapeutic anticoagulants can also show this interference effect. In all of these settings, factor activity increases in the assay as the plasma is increasingly diluted. Factor activity is underestimated when the plasma is diluted less, and a more accurate activity result is obtained when the test plasma is diluted more. **CB**

Recommendation 3.2.14:

In populations where lupus anticoagulant occurs, the WFH recommends the use of an APTT reagent insensitive to lupus anticoagulant to perform one-stage FVIII/FIX assays. **CB**

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Recommendation 3.2.15:

For all one-stage FVIII/FIX assays, only the clotting times of test sample dilutions that are within the range covered by the calibration curve should be used to calculate FVIII/FIX activity in the test sample.

• REMARK: When assaying test samples from patients with moderate or severe hemophilia A or B, an extended or additional calibration curve may be needed. It is not acceptable to extend the calibration curve by extrapolation without analyzing additional dilutions of the reference/calibration plasma. **CB**

Recommendation 3.2.16:

For all types of FVIII and FIX assays, an internal quality control (IQC) sample should be included with each batch of test samples analyzed. Results should only be released for patient management purposes after confirmation that the IQC result is within the target range for that material.

• REMARK: A description of how to set target ranges for IQC materials and handle out-of-range IQC results is available in the WFH laboratory manual. **CB**

Recommendation 3.2.17:

For internal quality control samples with FVIII/FIX activity in the range of 50-150 IU/dL, the between-assay coefficient of variation should be less than 10%.

• REMARK: Some studies have shown use of a stored calibration curve to be associated with higher between-assay CVs than use of a new calibration curve generated alongside patient samples. **CB**

Recommendation 3.2.18:

For monitoring replacement therapy with FVIII or FIX concentrates, the WFH recommends that laboratories use a FVIII/FIX assay that has been validated for use with the specific concentrate used for treatment.

• REMARK: This recommendation is particularly important for modified molecular forms of FVIII and FIX. **CB**

Recommendation 3.2.19:

For monitoring replacement therapy with plasma-derived FVIII concentrates, the WFH recommends use of a one-stage or chromogenic FVIII assay calibrated with a plasma standard traceable to a WHO international standard. **CB**

Recommendation 3.2.20:

For monitoring replacement therapy with clotting factor concentrates containing full-length recombinant FVIII, the WFH recommends use of a one-stage or chromogenic FVIII assay calibrated with a plasma standard traceable to a WHO international standard. **CB**

Recommendation 3.2.21:

For monitoring replacement therapy with efmoroctocog alfa (recombinant FVIII fused with human immunoglobulin G1 [rFVIIIFc]; Elocta®/Eloctate®), the WFH recommends use of a one-stage or chromogenic FVIII assay calibrated with a plasma standard traceable to a WHO international standard. **CB**

Recommendation 3.2.22:

For monitoring replacement therapy with turoctocog alfa pegol (recombinant B-domain-truncated FVIII with a site-specific 40-kDa polyethylene glycol group [N8-GP]; Esperoct®), the WFH recommends use of a chromogenic FVIII assay or APTT-based one-stage FVIII assay with validated reagents, including some ellagic acid activator reagents (Actin®, Actin® FS, SynthAFax™, DG Synth™) and some silica activator reagents (Pathromtin® SL, SynthASil™), calibrated with a plasma standard traceable to a WHO international standard.

• REMARK: One-stage FVIII assays with APTT-SP™, STA®-PTT Automate, or TriniCLOT™ APTT HS reagents significantlyunderestimate true FVIII activity of N8-GP and should not be used. **CB**

Recommendation 3.2.23:

For monitoring replacement therapy with damoctocog alfa pegol (recombinant B-domain-deleted FVIII with a site-specific 60 kDa branched polyethylene glycol group [BDD-rFVIII]; Jivi®), the WFH recommends use of a chromogenic FVIII assay or APTT-based one-stage FVIII assay with validated reagents, including the ellagic acid activator reagent Actin ® FSL and some silica activator reagents (Pathromtin® SL, SynthASil™), calibrated with a plasma standard traceable to a WHO international standard.

• REMARK: One-stage FVIII assays with the ellagic acid activator reagent Actin® FS or the kaolin activator reagent C.K. Prest® significantly overestimate true FVIII activity and should not be used. One-stage FVIII assays with APTT-SP™ and STA®- PTT Automate reagents significantly underestimate true FVIII activity and should not be used. **CB**

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Recommendation 3.2.24:

For monitoring replacement therapy with rurioctocog alfa pegol (full-length recombinant FVIII with non-site-specific 20-kDa polyethylene glycol; Adynovate®/Adynovi®), the WFH advises that more laboratory assay studies are required to inform recommendations about laboratory monitoring.

• REMARK: There are conflicting findings in the literature assessing the use of one-stage and chromogenic FVIII assays in samples containing rurioctocog alfa pegol. **CB**

Recommendation 3.2.25:

For monitoring replacement therapy with lonoctocog alfa (single-chain recombinant FVIII [rVIII-SingleChain]; Afstyla®), the WFH recommends use of a chromogenic FVIII assay calibrated with a plasma standard traceable to a WHO international standard.

• REMARK: The summary of product characteristics recommends chromogenic assays. It also states that the one-stage FVIII assay result underestimates the FVIII activity level by approximately 45% compared to the chromogenic assay result, and suggests that if the one-stage assay is used, the result should be multiplied by a factor of 2. **CB**

Recommendation 3.2.26:

For monitoring replacement therapy with plasma-derived FIX concentrates, the WFH recommends use of a one-stage or chromogenic FIX assay calibrated with a plasma standard traceable to a WHO international standard. **CB**

Recommendation 3.2.27:

For monitoring replacement therapy with clotting factor concentrates containing unmodified recombinant FIX, the WFH recommends use of a one-stage FIX assay calibrated with a plasma standard traceable to a WHO international standard.

• REMARK: Chromogenic FIX assays have been reported to underestimate the FIX activity of recombinant FIX concentrate. **CB**

Recommendation 3.2.28:

For monitoring replacement therapy with eftrenonacog alfa (recombinant FIX fused with human immunoglobulin G1 [rFIXFc]; Alprolix®), the WFH recommends use of a chromogenic FIX assay or APTT-based one-stage FIX assay with validated reagents, including some ellagic acid activator reagents (Actin®, Actin® FS, Actin® FSL), some silica activator reagents (Pathromtin® SL, SynthASil™), and a polyphenol activator reagent (Cephascreen®), calibrated with a plasma standard traceable to a WHO international standard.

• REMARK: One-stage FIX assays with STA®-PTT Automate or kaolin activator (C.K. Prest®) reagents significantly underestimate true rFIXFc (Alprolix®) activity and should not be used. **CB**

Recommendation 3.2.29:

For monitoring replacement therapy with albutrepenonacog alfa (recombinant FIX fused with recombinant human albumin [rFIX-RFP]; Idelvion®), the WFH recommends use of an APTTbased one-stage FIX assay with validated reagents, including some silica activator reagents (Pathromtin® SL, SynthASil™), calibrated with a plasma standard traceable to a WHO international standard. **CB**

• REMARK: One-stage FIX assays with the ellagic acid activator reagent Actin® FS or the kaolin activator reagent C.K. Prest® significantly underestimate true rFIX-RFP (Idelvion®) activity and should not be used. One-stage assays with the ellagic acid activator SynthAFax™ reagent or chromogenic FIX assays significantly overestimate true rFIX-RFP (Idelvion®) activity and should not be used. **CB**

Recommendation 3.2.30:

For monitoring replacement therapy with nonacog beta pegol (recombinant FIX with a 40-kDa polyethylene glycol moiety [N9-GP]; Refixia®/Rebinyn®), the WFH recommends use of a chromogenic FIX assay or APTT-based one-stage FIX assay with validated reagents, including the ellagic acid activator reagent SynthAFax™ or the polyphenol activator Cephascreen®, calibrated with a plasma standard traceable to a WHO international standard .

• REMARK: Most one-stage FIX assays significantly overestimate or underestimate true FIX activity of N9-GP and should not be used. One-stage assays using the ellagic acid activator reagent SynthAFax™ or the polyphenol activator reagent Cephascreen®, are suitable for monitoring therapy with N9-GP. **CB**

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Recommendation 3.2.31:

For patients receiving emicizumab in whom confirmation of expected emicizumab levels is required, the WFH recommends use of a modified one-stage assay including an additional pre-dilution step of test plasma and assay calibration with specific emicizumab calibrators.

• REMARK: Even at subtherapeutic levels of emicizumab, APTT may be normal or subnormal in patients with severe hemophilia A with or without inhibitors. **CB**

Recommendation 3.2.32:

For determination of FVIII activity in patients with hemophilia A receiving emicizumab, the WFH recommends use of a chromogenic FVIII assay containing bovine FX.

• REMARK: At therapeutic levels, emicizumab affects any chromogenic FVIII assay containing FX of human origin. Emicizumab may also affect chromogenic FVIII assays containing FIXa of human and FX of bovine origin but only at emicizumab levels higher than those expected in patients receiving recommended doses. **CB**

Recommendation 3.2.33:

For determination of FVIII inhibitor levels in patients receiving emicizumab, the WFH recommends use of a chromogenic FVIII assay containing bovine FX. **CB**

Recommendation 3.2.34:

For patients with a suspected neutralizing anti-emicizumab antibody, the WFH recommends measuring emicizumab levels using a modified one-stage assay including an additional pre-dilution step of test plasma and assay calibration with specific emicizumab calibrators.

• REMARK: Validated anti-drug antibody assays may also be used for this purpose, if available. **CB**

Recommendation 3.2.35:

For determination of anti-FVIII inhibitors in a sample containing greater than 5 IU/dL FVIII activity, the WFH recommends that prior to testing, the sample be heated to 56°C for 30 minutes and centrifuged at ambient temperature for a minimum of 1700 g for at least 5 minutes.

• REMARK: The quantification limit of the Nijmegen-Bethesda FVIII inhibitor assay is around 0.6 BU/mL.

• REMARK: The Nijmegen-Bethesda FVIII inhibitor assay requires use of buffered pooled normal plasma as a source of FVIII, which is then mixed with an equal volume of FVIII-deficient plasma to prepare the control mixture. **CB**

Recommendation 3.2.36:

For determination of anti-FIX inhibitors in a sample containing greater than 5 IU/dL FIX activity, the WFH recommends that prior to testing, the sample be heated at 56°C for 30 minutes and centrifuged at ambient temperature for a minimum of 1700 g for at least 5 minutes. **CB**

Recommendation 3.2.37:

For quantification of anti-FVIII inhibitors, the WFH recommends that the Nijmegen-Bethesda assay be used.

• REMARK: Bethesda assays detect neutralizing antibodies. A small proportion of anti-FVIII antibodies are non-neutralizing, shorten the half-life of infused FVIII, and are not detected by Bethesda assays.

• REMARK: The Nijmegen modification describes a specific method for buffering pooled normal plasma; other buffering methods may be suitable. **CB**

Recommendation 3.2.38:

For quantification of FVIII and FIX inhibitors, the WFH recommends that only residual FVIII/FIX activity between 25% and 75% of the FVIII/FIX in the control mixture be used to calculate inhibitor concentrations.

• REMARK: The most accurate inhibitor results are obtained when the residual FVIII/FIX activity is close to 50% of the level in the control mixture. **CB**

Recommendation 3.2.39:

For quantification of low-titer anti-FVIII inhibitors (<2 BU/mL), the WFH recommends use of a chromogenic Nijmegen-Bethesda FVIII assay to measure residual FVIII activity.

• REMARK: Use of a chromogenic Nijmegen-Bethesda FVIII assay instead of a one-stage FVIII assay provides greater specificity and reduces possible variability in measurement of residual FVIII leading to underestimation to the extent that a false positive inhibitor is reported when no inhibitor is present. **CB**

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Recommendation 3.2.40:

• For quantification of FVIII activity in recipients of gene transfer, the WFH advises that more research is necessary to determine the relative accuracy of chromogenic and one-stage assays in predicting hemostatic protection.

• REMARK: The one-stage assay appears to consistently produce FVIII activity results that are approximately 1.6-fold greater than those obtained with the chromogenic assay for multiple FVIII transgene products. Correlation with both plasma and recombinant FVIII-specific activity and clinical response may be needed for accurate determination of FVIII activity in recipients. **CB**

Recommendation 3.2.41:

For quantification of FIX activity in recipients of gene transfer, the WFH advises that more research is necessary to determine the relative accuracy of chromogenic and one-stage assays in predicting hemostatic protection.

• REMARK: FIX Padua (R338L) has been utilized for FIX gene therapy because it has a higher specific activity than native FIX. The one stage assay appears to consistently produce FIX Padua activity results that are approximately 1.6-fold greater than those obtained with the chromogenic assay. Correlation with both plasma and recombinant FIX-specific activity is needed for accurate determination of FIX Padua activity in recipients. **CB**

3.4 | Quality Assurance

Recommendation 3.4.1:

The WFH strongly recommends that coagulation laboratories implement quality assurance programs for all laboratory systems to ensure quality adherence and the reliability of laboratory blood testing procedures and reporting for the diagnosis and treatment of hemophilia. **CB**

Recommendation 3.4.2:

For hemostasis screening tests, the WFH recommends performing internal quality controls with at least two levels of internal quality control samples (normal and abnormal plasma samples) for all test batches at least daily. **CB**

Recommendation 3.4.3:

The WFH strongly recommends that clinical laboratories routinely participate in external quality assessment for each assay used for the diagnosis and treatment of hemophilia.

• REMARK: Participation in the WFH International External Quality Assessment Scheme (IEQAS) enables laboratories to improve and standardize laboratory testing for hemophilia. **CB**

CB, consensus based; PT, prothrombin time; APTT; activated partial thromboplastin time; PPP, platelet poor plasma; IQC internal quality control.

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For more information on the WFH Guidelines for the Management of Hemophilia, visit www.WFH.org/TGResourceHub