3 LABORATORY DIAGNOSIS AND MONITORING

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All statements identified as recommendations are consensus based, as denoted by CB.

3.1 | Introduction

- Different bleeding disorders may have very similar symptoms; therefore, a correct diagnosis is essential to ensure that a patient receives the appropriate treatment.
- An accurate diagnosis can only be made with the support of a comprehensive and reliable laboratory service. This is dependent on the laboratory following strict protocols and procedures, which require:
  - knowledge and expertise in coagulation laboratory testing;
  - use of the correct equipment and reagents; and
  - quality assurance (QA).
- For detailed information on technical aspects and specific instructions on screening tests and factor assays, please consult *Diagnosis of Hemophilia and Other Bleeding Disorders: A Laboratory Manual*, current edition, published by the World Federation of Hemophilia (WFH).[1]

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

Principles of diagnosis

- Diagnosis of hemophilia is based on the following three principles:
  - understanding the clinical features of hemophilia and the appropriateness of the clinical diagnosis;
  - using screening tests such as prothrombin time (PT) and activated partial thromboplastin time (APTT) or platelet function tests to identify the potential cause of bleeding (keeping in mind that normal screening test results do not exclude the possibility of a clinically relevant bleeding disorder being present); and
  - confirming the diagnosis by factor assays and other appropriate specific investigations.

Technical aspects

Preparation of the patient prior to taking a blood sample

- Fasting is not necessary before collection of blood for investigation of possible bleeding disorders.
- Whenever possible, patients should avoid medications that can affect test results such as acetylsalicylic acid (ASA), which can severely affect platelet function for 7-10 days.

- Levels of factor VIII (FVIII) and von Willebrand factor (VWF) may be temporarily elevated by strenuous exercise,[2] stress,[3] or inflammation enough to affect the accuracy of diagnosis. Factor VIII/ VWF levels increase during pregnancy.[4]

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

Sample collection

- The blood sample should be collected as per standard guidelines.[5]
- The sample should preferably be collected near the laboratory to ensure quick transport, and it should remain capped during transport.
- Results of tests can change according to the interval between collection and testing and according to sample storage conditions.[6-8] Higher temperatures (>25°C) lead to loss of FVIII activity over time,[9] whereas cold storage (2-8°C) may lead to cold activation of several proteolytic systems.[7,10] Storage of blood samples before processing at 2-8°C can lead to loss of FVIII and VWF sufficient to cause unaffected patients to be misdiagnosed with von Willebrand disease (VWD).[11]
- Specific guidance is available in relation to sample collection.[10] Venipuncture must be aseptic, and the sample must be collected within 1 minute of tourniquet application without prolonged venous stasis.
- Blood should be withdrawn into a plastic syringe or an evacuated collection system. The needle should be 19-21 gauge for adults and 22-23 gauge for small children. Collection through peripheral venous catheters or non-heparinized central venous catheters can be successful for many hemostasis tests.[10,12]
- Blood from an indwelling catheter should be avoided for some coagulation tests, particularly if platelet aggregation testing is being performed.
- Frothing of the blood sample should also be avoided. It is only necessary to discard the first 2 mL of blood collected if blood is collected through a catheter.[10]
- The sample should be collected in citrate tubes containing 0.105M-0.109M (c3.2%) aqueous trisodium citrate dihydrate, maintaining the proportion of blood to citrate at a 9:1 ratio. If the tube contains less than 90% of the target volume, results may be adversely affected, and prolongation of PT and APTT is expected when tubes contain less than 80% of target volume.[10]
- Patients with an elevated hematocrit above 55% have a reduced plasma volume leading to an exponential increase in PT and APTT with increasing hematocrit, which can be avoided by adjusting the ratio of blood to anticoagulant.[13,14]
- Results of some PT and APTT tests are different if samples are collected into 3.8% trisodium citrate.[10] The sample should be mixed promptly and adequately with citrate solution by gentle inversion 3 or 4 times.[10]
- If platelet-poor plasma (PPP) is frozen for future testing, the storage conditions affect the stability of the frozen material.[7] If the sample is frozen at −70°C, it may be stored for up to six months.[7,15] Storage at −20°C is usually inadequate.
- Frozen samples must be thawed rapidly in a water bath for 4-5 minutes at 37°C to avoid formation of cryoprecipitate.

Preparation of platelet-poor plasma (PPP)

- Most coagulation tests require the use of PPP.
- PPP should be prepared as per standard guidelines.[5,7]
- The residual platelet count in PPP depends on the centrifugation conditions including adverse effects on platelet function testing if refrigerated centrifuges are used since cold can activate platelets.[7,10]
- PPP may be kept at room temperature (20-25°C) prior to testing.
- Plasma that has been hemolyzed during collection and processing should not be used for platelet function testing, APTT testing, or related testing, irrespective of which method and instrument are used for analysis.[7,16,17] PT and fibrinogen testing are less affected, and only gross in vitro hemolysis may be relevant.[10,16] Adding hemolysate to plasma in vitro may give misleading results.[16,18]
- Sample acceptance criteria should take into account the risks from rejection (and delayed or missing test results)

against the risks of acceptance and testing (and the degree to which sample artefacts may or may not influence clinical management).

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**

Endpoint detection

- Many laboratories now have some form of semior fully automated coagulation analyzers. Accurately detecting the clotting endpoint using a manual technique requires considerable expertise, particularly if clotting time is prolonged or if fibrinogen concentration is low, and the clot is thin and wispy.
- For manual testing, the tube should be tilted 3 times every 5 seconds through an angle of approximately 90° during observation. The tube should be immersed in a water bath at 37°C between tilting.

Screening tests

- Platelet count, PT, and APTT may be used to screen a patient suspected of having a bleeding disorder.[19]
- The sensitivity of both PT[20] and APTT tests[21,22] to factor deficiencies are influenced by the type of reagents used to perform the test.

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**

TABLE 3-1 Interpretation of screening tests

Possible diagnosis	PT	APTT	Platelet count
Normal	Normal	Normal	Normal
Hemophilia A or B	Normal	Prolonged[a]	Normal
VWD	Normal	Normal or Prolonged[a]	Normal or reduced
Platelet defect	Normal	Normal	Normal or reduced

Abbreviations: APTT, activated partial thromboplastin time; PT, prothrombin time; VWD, von Willebrand disease.
[a]The same pattern can occur in the presence of FXI, FXII, prekallikrein, or high molecular weight kininogen deficiencies.

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

- Bleeding time testing lacks sensitivity and specificity, and it is also prone to performance-related errors. Therefore, other tests of platelet function such as platelet aggregometry are preferred when available.[23,24]
- Based on the results of these tests, the category of bleeding disorder may be partially characterized to guide subsequent analysis (see Table 3-1).
- These screening tests may not detect abnormalities in patients with mild bleeding disorders, including some variants of VWD, some cases of genetically confirmed mild hemophilia A or B, defects of platelet function, FXIII deficiency, and those rare defects of fibrinolysis which may be associated with a bleeding tendency.

Correction studies

- Abnormal screening tests may be further investigated using correction or mixing studies.
- Correction or mixing studies using pooled normal plasma (PNP) may help to define whether prolonged coagulation times are due to factor deficiency or circulating anticoagulants or inhibitors.
- The APTT of a patient/normal plasma mix may initially be normal and then progressively prolonged on incubation in the presence of a time-dependent inhibitor (e.g., many acquired autoantibodies against FVIII), although this pattern can be variable in cases with complex kinetics.
- Correction studies with FVIII/FIX-deficient plasma may be used to identify the particular deficiency if a factor assay is not available.

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

Factor assays

- Several types of FVIII assay including chromogenic and fluorogenic clotting assays are available.[25-30] One-stage clotting assays based on APTT are the most commonly used techniques in most regions.[26,27]
- FVIII- and FIX-deficient plasma must completely lack FVIII and FIX, respectively, i.e., it must contain < 1 IU/dL and have normal levels of other clotting factors.[1]
- The level of clotting factors in pooled normal plasma varies substantially between pools,[31,32] therefore, a system of international units (IUs) has been established for continuity and traceability.[31,33] Factor levels are reported in international units, either per mL or per decilitre (IU/dL). If IU/dL is used, then results are not interchangeable with percentage (%) of pooled normal plasma.[34]
- Use of a single test plasma dilution leads to assay inaccuracy in the presence of some inhibitors, including lupus anticoagulants (LA),[35] specific high-responding factor inhibitors, and some anticoagulant drugs,[36] and leads to assay imprecision.
- Assay calibration method can affect the quality of results.[37,38] When assaying test samples from patients with moderate or severe hemophilia, an extended or separate calibration curve may be needed. It is not acceptable to simply extend the calibration curve by extrapolation without analyzing additional dilutions of the calibration plasma.
- Some cases of genetically confirmed mild hemophilia A show normal FVIII activity when a one-stage assay is

used for diagnosis but reduced activity in chromogenic and two-stage clotting assays.[39-46] The reverse can also occur.[40,47,48] This means that more than one type of FVIII assay is needed to detect all forms of mild hemophilia A.
- All patients with reduced FVIII activity and a possible diagnosis of hemophilia A should have a full laboratory assessment to rule out VWD. This is especially important to differentiate VWD Normandy from mild hemophilia A since both have a normal level of VWF antigen usually associated with a reduced FVIII activity.[49]
- Chromogenic FIX assays are becoming more available,[50-54] and one study has reported that a chromogenic FIX assay may correlate better with the clinical picture than a one-stage assay in some hemophilia B cases.[53]
- Thrombin generation tests have been used in characterizing hemophilia[55-57] but are not in widespread use.

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**

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**

Post-FVIII/FIX infusion monitoring

- Lower than expected recovery and/or reduced half-life of infused clotting factor concentrates (CFCs) may be an early indicator of the presence of inhibitors.
- For samples containing FVIII or FIX CFCs, results of FVIII or FIX assays may vary according to whether a one-stage or chromogenic assay is used for analysis and sometimes according to the specific reagents or kits used in the assay.
- If factor assays are used to confirm efficacy of treatment or to make dose adjustments, bear in mind that some assays are unsuitable for monitoring some products.[58]
- Using an assay that markedly overestimates activity compared to the expected results from the labelled potency of the concentrate could lead to undertreatment and clinical risk.
- A full consensus on the tolerable degree of difference in results from different assays before patient management is adversely affected has not been established at the time of this writing; in the meantime, assays that give results that differ by more than 25-30% from the labelled potency of the concentrate vial are best avoided or, in any case, should not be used without taking account of such differences.
- Routine in-house assays can be used for post-infusion monitoring, provided that the local assay system (method and reference/ calibrator) is included in the manufacturer's guidance.[59] Any local assay should be verified for use with the specific CFC being used.[60]
- A number of articles have reviewed the published evidence related to use of specific assays for monitoring specific extended half-life (EHL) and unmodified CFCs.[58,60,61]
- One-stage assays used to monitor the single-chain recombinant FVIII molecule lonoctocog alfa (Afstyla®) underestimated relative potency by 45% whereas chromogenic assay recovered the expected values[62] which led to a recommendation that chromogenic assay is preferred, and that one-stage assay results should be multiplied by a conversion factor of 2 to determine the patient's FVIII activity level.[63] Such an approach did not fully correct for reagent differences,[64] and some experts have specifically recommended against using an assay known to give discrepant values and multiplying the result by a correction factor in this way.[65] Since there may be lot-to-lot variation in reagents used for factor assays, any such conversion factor should be verified for the lot numbers in use.
- There are numerous published assay studies comparing results in samples containing CFCs including EHL FVIII and FIX concentrates. Despite this, there are a number of one-stage and chromogenic assay reagents that have not been studied for use with some CFCs at the time of this writing. The reader is referred to the references in Table 3-2 (FVIII) and Table 3-3 (FIX) to see the evidence supporting the recommendations below.

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. C**

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**

TABLE 3-2 Publications with data related to the use of different FVIII assays in the presence of recombinant and modified factor VIII concentrates

Product type	Brand name	International non-proprietary name	References
Full-length recombinant	Advate®, Kogenate® FS, Kovaltry®	Octocog alfa	Church (2018)[66], Kitchen (2016)[67], Kitchen (2016)[68], Turecek (2016)[69]
BDD FVIII	NovoEight®	Turoctocog alfa	Viuff (2011)[70]
BDD FVIII	ReFacto AF®	Moroctocog alfa	Kitchen (2016)[68], Jacquemin (2018)[71], Cauchie (2013)[72], Morfini (2003)[73], Ingerslev (2004)[74], Santoro (2009)[75]
BDD FVIII fused to Fc portion of IgG1	Elocta®/Eloctate®	Efmoroctocog alfa	Powell (2012)[76], McCue (2015)[77], Sommer (2014)[78], Kitchen (2019)[79]
B-domain-truncated FVIII with site-specific 40 kDa polyethylene glycol moiety	Esperoct®	Turoctocog alfa pegol	Hillarp (2017)[80], Pickering (2016)[81], Persson (2019)[82], Ezban (2019)[83], Hegemann (2019)[84], Tiefenbacher (2019)[85]
BDD FVIII with site-specific 60 kDa polyethylene glycol	Jivi®	Damoctocog alfa pegol	Church (2018)[66], Gu (2014)[86]
Full-length recombinant FVIII with non-site-specific 20 kDa pegylation	Adynovate®/Adynovi®	Rurioctocog alfa pegol	Turecek (2016)[69], Bulla (2017)[87], Weber (2017)[88]
Single-chain recombinant FVIII	Afstyla®	Lonoctocog alfa	St Ledger (2018)[62], Bowyer (2017)[64]
Recombinant BDD porcine FVIII	Obizur®	Susoctocog alfa	Turecek (2016)[69], Vanguru (2018)[89]

Note: Therapeutic products are denoted by both their international non-proprietary name and their brand name because of the latter' s more common usage and recognition by the community.

Abbreviations: BDD, B-domain- deleted; FVIII, factor VIII; kDA, kilodalton.

TABLE 3-3 Publications with data related to the use of different FIX assays in the presence of recombinant and modified factor IX concentrates

Product type	Brand name	International non-proprietary name	References
Recombinant	Not identified	Not identified	Wilmot (2014)[90]
Recombinant FIX fused to Fc portion of IgG1	Alprolix®	Eftrenonacog alfa	Kershaw (2018) [54], Sommer (2014)[91], Bowyer (2019)[92]
Recombinant fusion protein linking FIX to albumin	Idelvion®	Albutrepenonacog alfa	Horn (2019)[51], Bowyer (2019)[92]
Recombinant FIX with site-directed 40 kDa pegylation	Refixia®/Rebinyn®	Nonacog beta pegol	Bowyer (2016)[52], Rosen (2016)[93], Tiefenbacher (2017)[94], Ezban (2019)[95]

Note: Therapeutic products are denoted by both their international non-proprietary names and their brand names because of the latter' s more common usage and recognition by the community.

Abbreviations: FIX, factor IX; IgG1, immunoglobulin G1; kDA, kilodalton.

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 significantly underestimate 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 APTTbased 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**

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-FP]; Idelvion®), the WFH recommends use of an APTT-based 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.**
- **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-FP (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-FP (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

Emicizumab
- Emicizumab is an engineered bispecific antibody that binds both human FIX/FIXa and FX/FXa and which is not regulated by the mechanisms that regulate FVIII but which acts as a FVIII mimetic.[96,97]
- The APTT is considerably shortened by emicizumab to within or below the reference range irrespective of reagents used, which means that emicizumab affects all APTT-based laboratory tests and assays.[98-100]
- Emicizumab significantly interferes in chromogenic FVIII assays utilizing human FIXa and FX but not those using FIXa and FX of bovine origin. Local verification is needed for chromogenic kits containing bovine FX and human FIXa.[98,99]
- Emicizumab can be measured and reported in µg/mL using a modified one-stage assay with higher test sample dilution (in assay buffer) and calibrated with emicizumab-specific calibrators.[99]

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

Inhibitor testing

- The most frequently encountered functional inhibitors of hemostasis are lupus anticoagulants, which are not directed against specific clotting factors and whose presence should be excluded prior to specific factor inhibitor testing.
- Results of APTT testing on mixtures of test and normal plasma can be difficult to interpret, particularly since in acquired hemophilia there may initially be a full correction of APTT even in the presence of a potent specific anti-FVIII antibody. If anti-FVIII antibody is present, the APTT of this mixture will be prolonged with incubation.
- Most FVIII inhibitors that develop secondary to replacement therapy in patients with hemophilia A show a characteristic pattern: the APTT of a patient/PNP mixture is intermediate, i.e., between the APTTs of the two materials, and it is further prolonged when the mixture is incubated at 37°C for 1-2 hours.
- Confirmation that an inhibitor is directed against a specific clotting factor requires a specific inhibitor assay.
- Quantification of the inhibitor titer is performed in the laboratory, preferably using the Nijmegen-modified Bethesda assay for FVIII inhibitor testing,[1] because this modification offers improved specificity and sensitivity over the original Bethesda assay.[101-109]
- The results of Bethesda inhibitor assays can be affected by the use of different dilutions of test sample before those dilutions are mixed with normal plasma.[110]
- For patients treated with FVIII or FIX, washout is no longer necessary if a heat neutralization modification of the Nijmegen-Bethesda assay is used, which inactivates FVIII/FIX in the sample to allow detection of the inhibitor.[109,111-113] This is not required if FVIII/FIX is <5 IU/dL in the test sample since this low level will not have a significant effect on inhibitor titer calculations.
- Different types of FVIII assays can be used to determine the FVIII during the Nijmegen-Bethesda inhibitor assay.[114-118] The protocol for the US national inhibitor program requires a chromogenic assay to be used when positive FVIII inhibitor results below 2.0 BU are observed.[108] If there is suspicion of lupus anticoagulant or if the sample contains therapeutic anticoagulants such as heparin or direct FXa or FIIa inhibitors, it may be useful to confirm inhibitor presence using a chromogenic assay to measure residual factor activity (instead of a one-stage assay).
- An inhibitor titer of ≥ 0.6 BU/mL should be considered clinically significant.[119,120]
- Some non-neutralizing anti-FVIII antibodies which are not detected by the Nijmegen-Bethesda assay may be clinically relevant because they may increase the clearance of FVIII and can be measured by ELISA.[121-128]

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

Gene therapy

- Discrepancies between results of one-stage and chromogenic assays have been reported after both FVIII and FIX gene therapy.
- Results of one-stage FVIII assays were approximately 1.65-fold higher[129] and 1.5-fold higher[130] than chromogenic assays for two different therapies with B-domain–deleted (BDD) FVIII, which is in contrast to CFC-containing BDD FVIII where chromogenic results are higher than one-stage assay results.[58,75]
- Results of one-stage FIX assays varied according to reagents used but were higher than results obtained in chromogenic FIX assays in patients who had received FIX gene therapy with a high specific activity FIX Padua variant.[131]

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

Trained personnel

- A laboratory scientist/technologist with an interest in coagulation must have an in-depth understanding of the tests in order to achieve accurate results.
- In some cases, it may be beneficial to have a laboratory scientist/ technologist who has had further training in a specialist centre.

3.3 Use of correct equipment and reagents

Equipment

- The basic laboratory equipment requirements include a 37°C ± 0.5°C water bath for rapid thawing of frozen samples and for performing manual tests on any samples where automated analysis has failed, and calibrated automated pipettes.
- Plastic and glass consumables used in coagulation testing should not be re-used.
- Automated coagulometers offer significant advantages over manual methods of some semi-automates including improved accuracy precision, repertoire and in some cases automatic detection of pre-analytical problems.

Selection of coagulometers

- Important considerations in the selection of coagulometers include:
  - test repertoire;
  - operational requirements including service and breakdown response;
  - throughput;
  - comparability between the results on the primary analyzer and any back-up methods;
  - compatibility with blood sample tubes and plasma storage containers in local use; and
  - safety.
- Information is required in relation to the performance characteristics of the system. This can be obtained from a variety of sources including the published literature and manufacturers' data, but it may also require some form of local assessment. Detailed guidance on selection and assessment of analyzers is available.[132,133]

Reagents

- It is good practice to ensure continuity of the supply of a chosen reagent, with attention paid to continuity of batches and long shelf life. This may be achieved by asking the supplier to batch hold for the laboratory, if possible.
- Different reagent brands may have different sensitivities and should not be run side by side, unless this is done for a specific purpose.
- A normal reference range should be defined for all methods. Practical guidance on this is published,[1] and for APTT must take into account the sample collection and processing conditions used locally.

3.4 | Quality assurance

- Quality assurance covers all aspects of the diagnosis process from sample taking, separation and analysis, and internal quality control (IQC) through to reporting of the result and ensuring that it reaches the appropriate clinician within an appropriate time.

Internal quality control

- Internal quality control is used to establish whether a series of techniques and procedures is being performed consistently over a period of time.
- IQC measures are taken to ensure that the results of laboratory investigations are reliable enough to assist clinical decision-making, monitor therapy, and diagnose hemostatic abnormalities.
- Graphical display of quality control results, for example in the form of Levey-Jennings charts, may facilitate review of trends in IQC results.

External quality assessment

- External quality assessment (EQA) helps to identify the degree of agreement between the local laboratory results and those obtained by other centres.
- The WFH International External Quality Assessment Scheme (IEQAS) is specifically designed to meet the needs of hemophilia treatment centres worldwide. This scheme includes analyses relevant to the diagnosis and management of bleeding disorders. Details of this scheme, which is operated in conjunction with the U.K. National External Quality Assessment Service (UK NEQAS) for Blood Coagulation in Sheffield, U.K., can be obtained from the WFH.[134]
- In order for a laboratory to attain a high level of testing reliability and to participate successfully in an external quality assessment program, the laboratory must have access to appropriate reagents and techniques and an appropriate number of adequately trained staff.

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

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.