PART 6 Hemophilia Lab Investigation

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TOPICS COVERED

- ✓ Assay of Factors VIII and IX: One-Stage and Chromogenic Methods
- ✓ Performing Factor Assays on Analyzers for Which the Software Uses a Single Dilution
- ✓ Bispecific Antibodies
- ✓ Post-FVIII and FIX Infusion Monitoring
- ✓ Gene Therapy
- ✓ Laboratory Aspects for Hemostatic Rebalancing Therapy Treatment

Assay of Factors VIII and IX: One-Stage and Chromogenic Methods: The laboratory diagnosis of hemophilia A or B is made by measurement of factor activity (Srivastava et al, 2020). The most commonly used methodology is the one-stage clotting assay based on the APTT. The one-stage assay for FVIII activity is described in this section. The assay is based on a comparison of the ability of dilutions of standard and test plasmas to correct the APTT of a plasma known to be totally deficient in FVIII but containing all other factors required for normal clotting. For factors IX, XI, and XII, the assay is essentially the same and is performed by substituting the relevant deficient plasma for FVIII-deficient plasma, and after selection of the appropriate reference plasma (Baker et al, 2020). A one-stage FVIII or FIX assay cannot be performed in the presence of bispecific antibodies such as emicizumab (Jenkins et al, 2020).

Reagents:

- ✓ Platelet-poor citrated test plasma
- ✓ Standard (reference/calibrator) plasma

The standard (reference) plasma used should be either a locally prepared plasma pool kept at -70°C or lower, or a commercial standard plasma. In either case, this reference plasma must be calibrated for clotting assay against the current international standard for FVIII or FIX in plasma. It is not acceptable to assume that a pooled normal plasma has 100 IU/dl.

- ✓ Internal quality control plasma (CLSI, 2016)
- ✓ FVIII-deficient plasma

This is available commercially or may be collected from a hemophilia donor under the following conditions:

- ✓ Level is less than 1 IU/dl
- ✓ No history of antibodies to FVIII
- ✓ Received no treatment for two weeks including extended-half life (EHL) or bispecific antibody therapy
- ✓ Normal liver function tests

Abnormal liver function could lead to a reduction in other clotting factors, which affect the specificity of the assay. This plasma can be stored in aliquots at -20°C or lower for approximately 1 month (Woodhams et al, 2001; Zhao et al, 2018). It is preferable to use FVIII/FIX-deficient plasma produced by immunodepletion of FVIII or FIX from normal plasma using a monoclonal antibody. This type of material is available commercially and has the advantage of enhanced viral safety compared with plasma sourced from patients with hemophilia who have been treated with plasma-derived products. However, not all immunodepleted

plasmas are found to be <1 IU/dl, and care should be taken to check this before use. Some experts hold the view that the presence of normal concentrations of VWF in FVIII/FIX-deficient plasma may be an advantage, and there is evidence to support this in relation to assays performed as part of inhibitor determinations (Verbruggen et al, 2001).

- ✓ APTT reagent that is sensitive to factor deficiencies (CLSI, 2016)
- ✓ Owren's buffered saline (OBS or glyoxaline buffer; see reagent session)
- ✓ 25 mM $CaCl_2$ (note that Werfen $CaCl_2$ supplied with SynthASil is 20 mM)

Method:

- ✓ Make 1/10 dilutions of standard, QC, and test plasma in buffered saline in plastic tubes. (If the test plasma is expected to have a very low level of FVIII, start at a 1/5 dilution.)
- ✓ Using 0.2 ml volumes, make doubling dilutions in OBS of standard, QC, and test plasma from 1/10 to 1/40 in plastic tubes. (Mix each dilution well before transferring to the next tube.) Plasma dilutions should be tested immediately after preparation. If room temperature exceeds 25°C, it may be necessary to keep dilutions on wet ice prior to testing.
- ✓ Pipette 0.1 ml of each standard dilution into a 75 x 10 mm glass tube.
- ✓ Add 0.1 ml of FVIII-deficient plasma and transfer to 37°C water bath.
- ✓ Add 0.1 ml of APTT reagent and incubate for 5 minutes.
- ✓ At 5 minutes, add 0.1 ml $CaCl_2$ and record the clotting time.
- ✓ A "blank" should also be set up using 0.1 ml of OBS in place of test plasma.

The clotting time of the blank should be longer than the time of 1% FVIII activity of standard from the calibration graph. If the time is shorter, this indicates that the substrate plasma is not totally deficient in FVIII and thus is not a suitable substrate plasma

Results: Plotting results requires double logarithmic or logarithmic/linear scale graph paper. The 1/10 dilution is arbitrarily assigned a value of 100%, the 1/20 dilution a value of 50%, and the 1/40 dilution a value of 25%. If used, the 1/5 dilution has a value of 200%. Straight lines, parallel to each other, should be obtained. Read off concentration of test sample as shown in Figure 7. In this example, the FVIII concentration in the test sample is 7% of that in the standard. If the standard has a FVIII concentration of 85 IU/dl, the test sample has a concentration of 85 IU/dl x 7% = 6 IU/dl. If the lines are not parallel, the assay should be repeated. Non-parallel lines may occur due to technical errors. If technical error has been eliminated, it may be due to the presence of an inhibitor, which may act specifically against FVIII or may be of the "lupus type", showing a converging pattern. Diverging lines are typical of an activated sample or presence of a DOAC (Baker et al, 2020).

Notes: If the test plasma FVIII (or FIX) concentration is close to zero (i.e. the clotting times of all dilutions are like the blank), non-parallel lines may occur. The presence of lupus anticoagulant can interfere with the phospholipids in APTT reagents and produce non-parallel factor assays (Ruinemans-Koerts et al, 2001). The normal range should be established locally but often has a lower limit of 50 to 65 IU/dl for both FVIII and FIX. The accurate monitoring of some EHL products by one-stage assay may affected by the APTT reagent used (Gray et al, 2020). See Part 6 on EHL. One-stage FVIII or FIX assays cannot be performed in the presence of bispecific antibodies such as emicizumab. These drugs artificially shorten the APTT. A short time to clot in a one-stage assay corresponds to a high factor activity (Jenkins et al, 2020; Bowyer et al, 2020; Bowyer et al, 2023). See Part 6 on bispecific antibodies. One-stage chromogenic FVIII discrepancy has been described in mild hemophilia A in some geographical areas and rarely for mild hemophilia B (Pouplard et al, 2009). If possible, a new diagnosis of mild hemophilia should have FVIII:C or FIX:C also measured by chromogenic assay Bowyer et al, 2018). One-stage FVIII assays can be used to measure recombinant porcine FVIII therapy Bowyer et al, 2022). There is a mutation in FIX, FIX Padua (p.R338L), reported to have eight-fold higher FIX activity than antigen (Simioni et al, 2009).

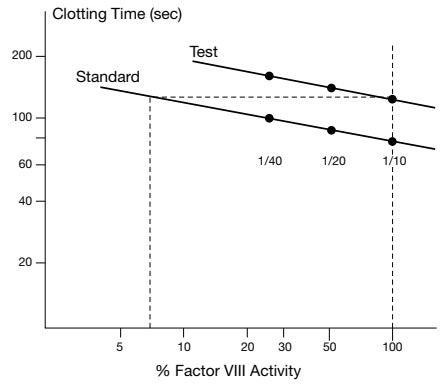

Figure 7. Graph of FVIII assay

Test samples should be assayed using three different sample dilutions as described above. This is a consistent recommendation in published national and international guidelines including those from WFH. It improves both assay accuracy and precision compared to use of a single test sample dilution. Factor assays are sometimes performed on analyzers with software designed for testing only a single test sample dilution. The following section describes a method that can be used to include three test sample dilutions on such analyzers.

Performing Factor Assays on Analyzers for Which the Software Uses a Single Dilution of Test Sample: The WFH recommends that one-stage APTT-based assays for FVIII and FIX should be done using three different dilutions of the test sample. This is described in the following document:

> WFH Guidelines for the Management of Hemophilia, 3rd edition. Srivastava A, Santagostino E, Dougall A, Kitchen S, Sutherland M, Pipe SW, Carcao M, Mahlangu J, Ragni MV, Windyga J, Llinás A, Goddard NJ, Mohan R, Poonnoose PM, Feldman BM, Lewis SZ, van den Berg HM, Pierce GF ; Chapter 3: Laboratory Diagnosis and Monitoring. Steve Kitchen, Francisco de Paula Careta, Silmara A de Lima Montalvao, Emna Gouider, Radoslaw Kaczmarek, Claude T. Tagny, Pierre Toulon, Glenn F. Pierce, Alok Srivastava. Haemophilia 2020; 26 (Suppl 1): 35-48.

The full document is available for free download on the WFH website via the following link:

Guidelines for the Management of Hemophilia - eLearning Platform (wfh.org)

The following text is reproduced from the WFH guideline:

Recommendation: For laboratory investigation due to clinical suspicion of hemophilia using one-stage FVIII/FIX assays, the WFH recommends analysis using 3 different dilutions of the reference and 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] x 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.

The above principles should also be applied to one-stage assays of other clotting factors (i.e. FII, FV, FVII, FX, FXI, and FXII). Some analyzers have software that permits more than one dilution of test sample. For these analyzers, the test sample is presented for analysis and the analyzer constructs the different dilutions, runs the assay, and calculates the activity of the clotting factor being assayed. Other analyzers have software that permits only a single dilution of test sample. The procedure below can be used on such analyzers so that the recommendations of the WFH can be followed with improved assay accuracy and precision.

The examples given are for FVIII assays but can be used for one-stage assays of other factors.

- ✓ The test samples are presented to the analyzer undiluted (i.e. without any pre-dilution by the operator).
- ✓ The test sample is pre-diluted 1 in 2 by the operator using the same assay buffer as the analyzer uses, which is often Owren's buffer, but may be a different buffer.
- ✓ This can be done in any plastic tubes or vials that can be presented to the analyzer for analysis and which do not cause activation of the test sample.
- ✓ The test sample is pre-diluted 1 in 4 using the same assay buffer as the analyzer uses (see points 2 and 3 above).
- ✓ This means the analyzer is presented with three different materials derived from the same test sample.
- ✓ The analyzer is then requested to run a FVIII assay on each of these three materials (i.e. test sample undiluted, test sample pre-diluted 1 in 2, and test sample pre-diluted 1 in 4).
- ✓ There is one FVIII result obtained on the undiluted test sample.
- ✓ There is another result obtained on the test sample that was pre-diluted 1 in 2. This should be multiplied by 2 to correct for the pre-dilution.
- ✓ There is another result obtained on the test sample that was pre-diluted 1 in 4. This should be multiplied by 4 to correct for the pre-dilution
- ✓ The three numbers are then compared by the operator.
- ✓ Usually, the three numbers are very close to each other. If so, the operator can calculate and report the mean of the three numbers as the FVIII activity of the test sample.
- ✓ Sometimes, the three numbers are not close together. This can occur if the sample contains interfering substances or if coagulation has been activated in the sample (i.e. perhaps due to difficulties during sample collection).
- ✓ The operator must decide whether it is safe to use the mean of the three different answers.
- ✓ The WFH recommends using a simple mathematical assessment to decide.
- ✓ This is done by calculating the CV of the three different results.
- ✓ The mean of the three different results can be safely used if the CV is <15%.
- ✓ Using the mean in this way improves the precision of the test.
- ✓ If the CV is higher than 15%, the operator has more decisions to consider.
- ✓ When the factor level is 10 to 15 IU/dl, the CV of the three dilutions is higher than when the factor activity is at higher levels. In these samples, a CV of 20% can be accepted.
- ✓ When the factor level is below 5 IU/dl, a pre-dilution of 1 in 4 may reduce the factor activity below the lower limit of quantification, depending on the reagents. In these samples, it is acceptable to test only the undiluted test sample and the test sample pre-diluted 1 in 2. In this case, the lab should report the mean of the two results (after multiplying the analyzer result of the 1 in 2 dilution by 2), without calculation of CV.
- ✓ For samples with >15 IU/dl FVIII, if the CV is >15% and the result on the undiluted plasma is lower than the results on the third dilution, this indicates there may be an interfering substance in the test sample. In this case, the result obtained for the 1 in 4 dilution (after multiplication of analyzer result by 4 to correct for pre-dilution) will be the most accurate (see examples below).
- ✓ Examples of interfering substances that can cause this falsely low result in the undiluted test samples, are inhibitors such as lupus anticoagulant, UFH, DTIs, or direct FXa inhibitors.
- ✓ Use of a single dilution can lead to falsely low results and grossly inaccurate assays.

Some examples are given below.

Table 12. Example 1-FVIII assay with manual pre-dilution

	FVIII activity recorded by analyzer	FVIII activity after correcting for pre-dilution	Comments
Test samples without pre-dilution	25 IU/dl (%)	25 IU/dl (%)	No pre-dilution by operator so result does not change
Test samples diluted 1 in 2 before analysis	13.5 IU/dl (%)	27 IU/dl (%)	Analyzer result multiplied by 2
Test samples diluted 1 in 4 before analysis	6 IU/dl	24 IU/dl (%)	Analyzer result multiplied by 4

✓ Mean of three results is 25.3 IU/dl (%).
✓ Standard deviation of the three results is 1.53.
✓ CV of the three results is 6.0%.
✓ CV is <15%, so result reported is **25.3 IU/dl (%)**.

Table 13. Example 2-FVIII assay with manual pre-dilution

	FVIII activity recorded by analyzer	FVIII activity after correcting for pre-dilution	Comments
Test samples without pre-dilution	62 IU/dl (%)	62 IU/dl (%)	No pre-dilution by operator so result does not change
Test samples diluted 1 in 2 before analysis	33 IU/dl (%)	66 IU/dl (%)	Analyzer result multiplied by 2
Test samples diluted 1 in 4 before analysis	13 IU/dl	52 IU/dl (%)	Analyzer result multiplied by 4

✓ Mean of three results is 60.0 IU/dl (%).
✓ Standard deviation of the three results is 7.2.
✓ CV of the three results is 12.0%.
✓ CV is <15%.
✓ Result reported is **60.0 IU/dl (%)**.

Table 14. Example 3-FVIII assay with manual pre-dilution

	FVIII activity recorded by analyzer	FVIII activity after correcting for pre-dilution	Comments
Test samples without pre-dilution	7.0 IU/dl (%)	7.0 IU/dl (%)	No pre-dilution by operator so result does not change
Test samples diluted 1 in 2 before analysis	3.0 IU/dl (%)	6.0 IU/dl (%)	Analyzer result multiplied by 2
Test samples diluted 1 in 4 before analysis	2.1 IU/dl	8.4 IU/dl (%)	Analyzer result multiplied by 4

✓ Mean of three results is 7.1 IU/dl (%).
✓ Standard deviation of the 3 results is 7.1.
✓ CV of the 3 results is 16.9%.
✓ CV is <20%.
✓ Result reported is **7.1 U/dl (%)**.

Table 15. Examples with interfering substances present, FVIII assay results are in IU/dl (%)

Interference	Without pre-dilution	Pre-diluted 1 in 2		Pre-diluted 1 in 4		Results used to calculate CV	CV
		Analyzer results	Operator corrected result	Analyzer result	Operator corrected result		
Direct thrombin inhibitor	46.5	34.0	68.0	18.5	74.0	46.5, 68.0, 74.0	23.0%
Rivaroxaban	64.0	45.2	90.4	29.1	116.4	64.0, 90.4, 116.4	29.0%
Lupus anticoagulant	25.3	19.3	38.6	16.2	64.8	25.3, 38.6, 64.8	46.8%

- ✓ Note that similar patterns occur in other one-stage assays such as FIX.
- ✓ All three have CVs >15%.
- ✓ The effects of such interfering substances are usually lower in the 1 in 4 dilution sample than in the undiluted sample or in the 1 in 2 pre-dilution sample.
- ✓ The result for the 1 in 4 sample may still be an underestimation, but is the closest to an accurate result of the three tests performed. It may also be useful to add a 1 in 8 pre-dilution as a fourth test in such cases.
- ✓ The reportable results from the examples in the table could be >74 IU/dl (%), >116.4 IU/dl (%), or >64.8 IU/dl (%).

All three test samples in this example have FVIII activity that is not reduced below the normal range. Confirming that activity is not reduced, is sometimes sufficient for safe patient management.

Chromogenic FVIII:C and FIX assays: The laboratory diagnosis of hemophilia A or B is made by measurement of factor activity (Srivastava et al, 2020). The most commonly used methodology is the one-stage clotting assay based on the APTT (OSA). There are limitations to the one-stage assay, including interference if lupus anticoagulant, direct oral anticoagulants (DOACs) or extended half-life hemophilia therapies, including bispecific antibodies, are present (Gray et al, 2020; Jenkins et al, 2020; Bowyer et al, 2021; Moser et al, 2021; Ruinemans-Koerts et al, 2010). More importantly, mild hemophilia A is not excluded by the finding of a normal FVIII:C, and rarely FIX:C level, by OSA (Pouplard et al, 2009). Several groups have reported that a subgroup of mild hemophilia A patients have discrepancy between the activity of FVIII as determined using different types of assays (Pavlova et al, 2014). More than 20% of mild hemophilia A patients are associated with discrepancy in which chromogenic activity is two-fold or lower than OSA and the bleeding phenotype is commensurate with the chromogenic substrate assay (CSA) (Bowyer et al, 2018). A reverse form of assay discrepancy can also occur with two or more-fold lower FVIII:C by OSA than CSA. Reports of bleeding are much lower in these patients (Bowyer et al, 2018; Bowyer et al, 2011). Examples of results in such patients are shown in Table 16.

Table 16. Examples of patients with genetically confirmed mild hemophilia A and assay discrepancies

Case	One-stage assay (IU/dl)	Chromogenic assay (IU/dl)
A	101	13
B	88	28
C	15	69
D	55	40
E	58	33
F	72	36
G	84	45

Based on these results, it is advantageous for all hemophilia centers to have a chromogenic FVIII assay available. CSA should be performed on subjects with normal APTT and one-stage FVIIII activity in the presence of a personal or family history consistent with mild hemophilia. FVIII CSAs were first introduced in the early 1980s (Rosen et al, 1984) and a number of manufacturers have commercial kits for chromogenic assay of FVIII. Many of these are suitable for diagnosis of hemophilia A in the presence of normal one-stage FVIII activity. A small number of FIX CSA have been available since the mid-2010s and are mostly limited to research or specialist hemostasis laboratories (Kershaw et al, 2018). One-stage chromogenic FIX discrepancy has been described in mild hemophilia B but the discrepancy appears to compromise the classification of severity, with patients changing between moderate and mild hemophilia B (Pouplard et al, 2009; Truedsson et al, 2020)

Principle of analysis for FVIII CSA: Many automated coagulometers have the ability to perform CSA but since these assays were originally performed manually using microtiter plates, it is still possible to use a manual method. In some (but not all) chromogenic assays, all the FVIII in the sample is activated by thrombin. Activated FVIII then accelerates the conversion of FX to FXa in the presence of activated FIX, phospholipids, and calcium ions. The FXa activity is assessed by hydrolysis of a p-nitroanaline substrate specific to FXa. The initial rate of release of p-nitroanaline (yellow color) measured at 405 nm is proportional to the FXa activity and thus to the FVIII activity in the sample. The results of the patient plasma and quality control sample are compared to the standard (reference/calibrator) plasma to quantify the CSA using the same principles as with the OSA (Baker et al, 2020). The proteins used in FVIII CSA kits may be human or bovine sourced. For measurement of endogenous FVIII:C, or standard or extended half-life FVIII therapy, the source of proteins does not affect the CSA. The source of proteins is important when bi-specific antibodies are present in the plasma (see section Part 6 on bispecific antibodies).

Principle of analysis for FIX CSA: In some (but not all) chromogenic FIX assays, all the FIX in the sample is activated by FXIa. Activated FIX then accelerates the conversion of FX to FXa in the presence of activated FVIII, phospholipids, and calcium ions. The FXa activity is assessed by hydrolysis of a p-nitroanaline substrate specific to FXa. The initial rate of release of p-nitroanaline (yellow color) measured at 405 nm is proportional to the FXa activity and thus to the FIX activity in the sample as described above.

Notes: If the test plasma FVIII (or FIX) concentration is close to zero (i.e. the optical density of all dilutions are similar to the blank), non-parallel lines may occur. The normal range should be established locally but often has a lower limit of 50–65 IU/dl in both FVIII and FIX. The FIX CSA may not accurately measure recovery of some extended half-life FIX products (Gray et al, 2020; Bowyer et al, 2022). Chromogenic assays can be used to measure mimetic effect in plasma containing bispecific antibodies (Jenkins et al, 2020; Bowyer et al, 2020; Bowyer et al, 2023). Bovine FX-containing FVIII CSA can be used to measure recombinant FVIII therapy in plasma also containing bispecific antibodies. If possible, a new diagnosis of mild hemophilia A or B should have FVIII:C or FIX:C also measured by chromogenic assay (Bowyer et al, 2018). Some caution should be exercised when using CSA to measure recombinant Porcine FVIII as underestimation may occur (Bowyer et al, 2022). FIX CSA may underestimate recombinant standard half-life FIX therapies (Nederlof et al, 2020).

Measurement of EHL FVIII and FIX molecules: Modifications to recombinant FVIII or FIX have been made to extend the in vivo half-life of the therapy by altering the conformation of the molecule. Extension can be made by addition of polyethylene glycol (PEG) moieties, covalent linkage of the heavy and light chains of FVIII, fusion to albumin or covalently fused to the fc (fragment crystallizable) portion of human IgG1. The post infusion monitoring of recombinant EHL FVIII or FIX concentrates is necessary for clinical management of the patient with hemophilia. A lower-than-expected response or reduced half-life can indicate the requirement for additional therapy or possible development of anti-drug antibodies. Laboratory studies conducted during the pharmaceutical trials of each EHL, highlighted issues with the accurate laboratory measurement of some molecules. Over or underestimation was reported with some EHL FVIII or FIX but this was dependent on methodology or the APTT used in OSA. The method of molecule modification is not predictive of the factor assay response such that accurate monitoring of the three PEGylated FVIII

molecules may not be possible with the same APTT reagents. For currently licensed EHL FVIII molecules, chromogenic FVIII activity assays are all considered suitable for accurate monitoring but for OSA this may be reagent dependent. For EHL FIX molecules, there is no single assay methodology or reagent that will accurately measure all three currently licensed concentrates. An ultra long half-life concentrate, rFVIII-FC-VWF-XTEN, efanesoctocog alfa (Altuviiio/Altuvoct), was granted regulatory approval in the USA in 2023 and Europe in 2024. Monitoring is recommended by FVIII OSA using a particular APTT reagent, Siemens Actin FSL. The prevalent APTT reagents, Siemens Actin FS and Werfen Synthasil over and underestimated efanesoctocog alfa respectively. Chromogenic FVIII assays overestimate by 2-3 times the expected activity (Pipe, 2009). It is therefore necessary to carefully assess whether the assays available in each hemostasis laboratory are suitable for accurate monitoring of each EHL used as replacement therapy in their center. Tables 17 and 18 give examples of EHL rFVIII and rFIX concentrates, how the potency of the product was assigned and whether OSA or CSA are acceptable for use in monitoring the post infusion activity

Table 17. EHL FVIII molecules

Name	Company	Molecule	Potency label	OSA	CSA	References
Adynovi/ Adynovate Rurioctocog alfa pegol	Takeda	FVIII 2 x 10 kDa PEG	CSA	Varied results	Yes	Turecek et al, 2016 Bulla et al, 2017
Afstyla Ionoctocog alfa	CSL Behring	Single chain BDD FVIII	CSA	Results are approximately half of CSA	CSA	Bowyer et al, 2017 St Ledger et al, 2018
Elocta/Eloctate efmoroctocog	Sobi	FVIII FC fusion	CSA	yes	Yes	Sommer et al, 2014 Powell et al, 2012 Pouplard et al, 2020
Esperoct Turoctocog alpha pegol	Novo Nordisk	BD truncated rFVIII 40 kDa PEG	CSA	Varied results	Yes	Pickering et al, 2016 Hillarp et al, 2017
Jivi Damoctocog alfa pegol	Bayer	BDD rFVIII 60 kDa PEG	CSA	Not silica or kaolin APTT reagents	Yes	Gu et al, 2014 Church et al, 2018
Altuvoct/Altuviio Efanesoctocog alfa	Sanofi	rFVIII FC-VWF-XTEN	OSA	Actin FSL recommended	Overestimates 2-3 fold	Pipi et al, 2024

Table 18. EHL FIX molecules

Name	Company	Molecule	Potency label	OSA	CSA	References
Alprolix etrenonacog	Sobi	rFIX FC fusion	OSA	Not some silica or kaolin APTT reagents	Yes	Sommer et al, 2014 Bowyer et al, 2019 Persson et al, 2018
Idelvion albutrepenonacog	CSL Behring	rFIX albumin fusion	OSA	Varied results	no	Persson et al, 2018 St Ledger et al, 2016 Kitchen et al, 2017 Horn et al, 2019 Pouplard et al, 2019
Refixia/Rebinyn Nonacog beta pegol	Novo Nordisk	rFIX 40 kDa PEG	OSA	Only Cephascreen and Synthafax validated	Yes	Bowyer et al, 2016 Tiefenbacher et al, 2017 Young et al, 2016

Bispecifific Antibodies:

Measurement of bispecific antibodies: Bispecific antibodies are a class of non-replacement therapy for hemophilia A. They act to bridge human FIXa and human FX, in the absence of FVIII, to promote activation of FX. Bispecific antibodies differ from native FVIII in a number of intrinsic aspects, including a lack of regulatory mechanisms, which impact hemostasis assays (Lenting et al, 2017). Future, more potent, generations of bispecific antibodies may have a greater impact on hemostasis testing (Bowyer et al, 2023).

APTT and bispecific antibodies: Bispecific antibodies do not require activation to participate in the activation of FX. In the presence of bispecific antibodies, the APTT is dramatically shortened, often to below the bottom of the reference range (Bowyer et al, 2020; Bowyer et al, 2023). The APTT is not sensitive enough to changes in bispecific antibodies concentration to use in the monitoring of these therapies, however a prolongation to the APTT in a patient with a previously short APTT may indicate a loss of efficacy or compliance (Druzgal et al, 2020; Valsecchi et al, 2021).

OSA and bispecific antibodies: In the presence of bispecific antibodies, standard, plasma-calibrated, APTT-based assays, including FVIII, FIX, FXI, XII, protein C, protein S, and activated clot time (ACT), overestimate the amount of clotting factor or natural inhibitor so are unsuitable for use (Bowyer et al, 2023; EMA, 2018).

Modified FVIII OSA and bispecific antibodies: Commercial, product-specific calibrator (standard/reference) and quality control plasmas are available for the first generation of bispecific antibodies. The one-stage FVIII assay can be modified using these product-specific calibrators alongside an increased plasma dilution (dilutions of 1/40 or 1/80 instead of 1/10) to measure drug concentration of bispecific antibody in µg/ml. This modified assay will also measure any endogenous or replacement FVIII present in the plasma (Bowyer et al, 2020).

Chromogenic FVIII and bispecific antibodies: FVIII CSA that contain human FX and FIXa are sensitive to the presence of bispecific antibodies and measure some "mimetic or surrogate" FVIII-like activity (Bowyer et al, 2020; Bowyer et al, 2023). Human CSA can be used as a marker of the presence of bispecific antibodies in patients receiving prophylaxis. This is not interchangeable with the drug concentration level detailed above. The human CSA will also measure any endogenous or replacement FVIII present in the plasma (Bowyer et al, 2020). FVIII CSA that contain bovine FX and either human or bovine FIXa are insensitive to the presence of first-generation bispecific antibodies but may demonstrate some sensitivity to next generation bispecific antibodies (Bowyer et al, 2020; Bowyer et al, 2023). Bovine FX CSA should

be used to measure any endogenous FVIII or replacement FVIII therapy and to measure residual FVIII in the Bethesda inhibitor assay as detailed below.

Bethesda inhibitor assay and bispecific antibodies: The measurement of residual FVIII following incubation in the Bethesda inhibitor assays is usually by OSA (Verbruggen B, 1995) although chromogenic and fluorogenic measurement have been validated (Miller et al, 2021). In the presence of bispecific antibodies, OSA cannot be used so the CSA must be used. It is important that the CSA kit used contains bovine FX and either human or bovine FIXa to exclude the effect of the bispecific antibody, otherwise the inhibitor titer will be underestimated (Bowyer et al, 2021; Miller et al, 2021).

Post-FVIII and FIX Infusion Monitoring: The post-infusion monitoring of plasma-derived or recombinant standard half-life FVIII or FIX concentrates is necessary for clinical management of the patient with hemophilia. A lower-than-expected response or reduced half-life can indicate the requirement for additional therapy or possible inhibitor development. Measurement of replacement therapy should ideally be performed using the same assay method and reagents that were originally used to assign potency to the product. If this is not possible then an alternative, validated assay, should be used. In Europe, potency labelling for FVIII concentrates is by CSA (Barrowcliffe et al, 2002) and for FIX concentrates is by OSA (Kitchen et al, 2016). The United States Food and Drug Administration (FDA, 2020) recommends the use of OSA for FIX concentrates however, some FVIII concentrates have potency assigned by OSA and some by CSA. The WFH recommends the use of a FVIII or FIX assay that has been validated for use with the specific concentrate used for treatment and is calibrated with a plasma standard traceable to the current WHO international standard (Srivastava et al, 2020). Other guidelines recommend the use of OSA or CSA calibrated with plasma standards can be used to monitor plasma derived FVIII concentrates unless there is evidence to the contrary and use of a OSA for FIX concentrates (Gray et al, 2020). Tables 19 and 20 give examples of commonly available plasma derived and recombinant standard half-life FVIII and FIX concentrates, how the potency of the product was assigned and whether OSA or CSA are acceptable for use in monitoring the post infusion activity.

Table 19. Plasma-derived concentrate examples

Concentrate	Factor	Potency assignment	CSA	OSA	References
Factane	VIII	CSA	Yes	Yes	Adcock et al, 2018
Octanate	VIII	CSA	Yes	Yes	
FVIII 8Y	VIII	CSA	Yes	Yes	
Haemoctin	VIII	CSA	Yes	Yes	
Octaplex	VIII	CSA	Yes	Yes	
Recombinate	VIII	CSA	Yes	Yes	Jennings et al, 2007
Fanhdi	VIII	CSA	Yes	Yes	Peyvandi et al, 2016
Hemofil M	VIII	CSA	Yes	Yes	Peyvandi et al, 2016
Emoclot	VIII	CSA	Yes	Yes	Peyvandi et al, 2016
Replenine	IX	OSA		Yes	
Betafact	IX	OSA Pathromtin SL (silica)	Likely acceptable	Yes	Adcock et al, 2018
Mononine	IX	OSA Pathromtin SL (silica)	Yes	Yes	Bowyer et al, 2016 Wilmot et al, 2014
Octafix	IX	OSA Pathromtin (Kaolin)	No data	Yes	
Alphanine	IX	OSA		Yes	Aznar et al, 2009

Table 20. Recombinant standard half-life concentrates examples

Concentrate	Factor	Potency assignment	CSA	OSA	References
Advate	FVIII-full length	CSA	Yes	Yes	Kitchen et al, 2016 Kitchen et al, 2019
Refacto AF	FVIII-BDD	CSA	Yes	Yes, with Refacto lab standard	Kitchen et al, 2016 Morfini et al, 2003 Ingerslev et al, 2004
Novo8	FVIII-BDD	CSA	Yes	Yes, but some overestimation at trough levels	EMA, 2021 Viuff et al, 2011
Nuwiq	FVIII-BDD	CSA	Yes	Yes	Lissitchkov et al, 2016 EMA, 2022 Klukowska et al, 2016 Tiefenbacher et al, 2019
Xyntha	FVIII-BDD	OSA	No-overestimation	Yes	FDA, 2020
Kovaltry	FVIII	CSA	Yes	Yes	Mahlangu et al, 2018 Kitchen et al, 2016
Benefix	IX	OSA	Insufficient data	Yes	Bowyer et al, 2016 Kershaw et al, 2018 Sommer et al, 2014
Rixubis	IX	OSA Pathromtin SL validated	Yes	Yes	Kershaw et al, 2018 Gritsch et al, 2014

Gene Therapy: Hemophilia A and B are monogenic disorders and therefore ideal candidates for gene manipulation. A varied range of gene therapy strategies, including gene editing, have been researched in humans for both disorders since the early 2000s (De Wolf et al, 2023). In the mid 2020s, regulatory approval was granted in some countries for the limited use of gene therapy products for treatment of persons with hemophilia A or B. The measurement of FVIII or FIX transgene expression is essential to determine duration of response and whether additional therapies are required to achieve hemostasis. To minimize inter-laboratory variability, it is sensible to restrict routine monitoring to a limited number of specialized hemostasis laboratories in each country. Clinical trials of several gene therapies have reported assay variability with chromogenic FVIII and FIX activities 1.5 to 3.0-fold lower than one-stage activities. Differences have been reported between reagents in the same assay method. Due the constraints of adequate plasma volume it is difficult to conduct multicenter laboratory comparison studies in samples from patients who received gene therapy. However, there are limited data available regarding measurement of the candidate FVIII or FIX molecules using a range of methods or reagents. Wherever possible, to minimize variability, the reagents and methods used in the pharmaceutical clinical trials should be used by laboratories for monitoring post gene therapy expression (Table 21).

Table 21. Gene therapy for hemophilia A and B

	Pharmaceutical company	Trade name	Assay method	Reagents
Hemophilia A				
Valoctocogene roxaparvovec	Biomarin	Roctavian	CSA	Coatest SP4
Hemophilia B				
Etranacogene dezaparvovec	CSL Behring	Hemgenix	OSA	Synthasil
Fidanacogene elaparvovec	Pfizer	Beqvez	OSA	Synthasil

Gene therapy for hemophilia A: All approaches for hemophilia A have used AAV vectors and B-domain deleted (BDD) recombinant FVIII. Phase 1–3 trials of Roctavian (AAV5-FVIII-SQ, Valoctocogene roxaparvovec, Biomarin) measured transgene FVIII:C with Coatest SP4 CSA and OSA using Siemens Actin FSL APTT reagent on Siemens BCS XP analyzer (Rangarajan et al, 2017). CSA FVIII:C were approximately half of OSA FVIII:C (Mahlangu et al, 2023). Rosen et al reported comparable results between Coatest SP4 and Hyphen Biomed CSA when measuring AAV5-FVIII-SQ (4). A two-center comparison of FVIII:C measured by OSA or CSA in plasma from patients following therapy with Roctavian reported an approximate 1.65-fold difference between OSA and CSA. Similar FVIII:C results were obtained with Hyphen Biomed CSA and two other CSA (Platton et al, 2024). The authors concluded that OSA were not suitable for measuring FVIII following gene therapy with Roctavian and only CSA should be used.

Gene therapy for hemophilia B: The two currently approved hemophilia B products, Hemgenix (Etranacogene dezaparvovec, CSL Behring) and Beqvez (Fidanacogene elaparvovec, Pfizer), both use a naturally occurring, highly active FIX variant, FIX-Padua (R338L) (Simioni et al, 2009). Pharmaceutical program have only reported FIX activity by OSA in their study reports. Variability between reagents and methodologies have been reported when measuring FIX-Padua following gene expression and in plasma spiked with the FIX-Padua molecule. A global field study of plasma spiked with a recombinant R338L molecule (FLT180a, verbrinacogene setparvovec, by Freeline Therapeutics which is currently paused at the end of phase 1/2 testing) reported a 3-fold difference in FIX activity between 15 different OSA and CSA. A 1.8-fold variation was observed across 13 APTT reagents in the OSA whilst results from both CSA were approximately half that of expected activity measured by Synthasil OSA (Foley et al, 2023). Measurement of Beqvez transgene FIX-Padua activity highlighted assay differences between five APTT reagents in the OSA and between OSA and CSA (Robinson et al, 2021). In a global field study using plasma from participants in the phase 1/2a gene therapy trial, FIX:C was higher with the silica-activated APTT reagent, Synthasil, in the OSA than with the ellagic acid-activated APTT reagents, Actin FS and Actin FSL, or CSA (Pittman et al, 2024). There is minimal laboratory data available for the measurement of FIX activity following Hemgenix gene therapy. The Summary of Product Characteristics states that FIX activity is lower by CSA than OSA (EMA, 2024). Phase 1–3 clinical trials FIX used Synthasil APTT reagent in the OSA and an undisclosed CSA; OSA FIX activities were at least two-fold higher than with CSA (Pipe et al, 2023; Miesbach et al, 2018).

Laboratory Aspects for Hemostatic Rebalancing Therapy Treatment: Non-factor replacement therapies for hemophilia A or B aim to promote coagulation and rebalance hemostasis by targeting natural anti-coagulants or inhibitors of coagulation including antithrombin, tissue factor pathway inhibitor (TFPI), protein C, or protein S (Nogami and Shima, 2023). Some of these therapies have been approved for use in certain patient groups, others are currently in pharmaceutical trials.

Molecules that target antithrombin: Heparin-activated antithrombin (AT) exerts inhibitory actions on thrombin, FXa, FIXa, FXIa, and FXIIa (Rezaie et al, 2020). A small interfering RNA molecule, fitusiran, which targets AT synthesis in hepatocytes, has been developed to improve thrombin generation (Young et al, 2023). In clinical trials an 82–87% reduction in AT was associated with increased thrombin generation (Pasi et al, 2021). Target AT activity levels are 15–35 IU/dl. AT assays are well established as part of

thrombophilia testing, but it is rare to measure such low AT activities. A global comparative laboratory field study assessing the measurement of a range of AT activities (9–100 IU/dl) concluded that some AT assays should not be used to monitor AT during fitusiran therapy (Chhabra et al, 2024).

Molecules that target TFPI: Anti-TFPI antibodies target the Kunitz 2 domain of TFPI and prevent binding to activated FX thus enabling FXa generation to continue (Mast et al, 2022). The first anti-TFPI monoclonal antibody (concizumab, Novo Nordisk, Denmark) was approved for use in 2023 in Canadian hemophilia B patients with inhibitors. An alternative anti-TFPI antibody, marstacimab (Pfizer, USA) is under consideration for approval in USA and Europe for people with hemophilia A or B without inhibitors (Matino et al, 2023). TFPI assays are available in some research or specialist laboratories, but the clinical utility of measurement is unclear.

Molecules that target activated protein C (APC): APC, in conjunction with cofactor protein S, inactivates FVa and FVIIIa to prevent further thrombin generation. FV Leiden is a p.Arg506Gln (c.1691G>A) mutation at the primary APC cleavage site in activated FV. The presence of FV Leiden slows the inactivation of FVa by APC and is the most common cause of thrombophilia in humans (Van Cott et al, 2016). Alternative approaches to target APC are in clinical trials. A humanized monoclonal antibody that inhibits activated protein C has been reported to restore hemostasis in hemophilic mice (Jiang et al, 2023) and a serine protease inhibitor (serpin) which only targets APC, not the precursor protein C, has commenced human trials (Baglin et al, 2023). Assays for protein C and APC are routinely available in many tertiary hemostasis laboratories should measurement be required for drug monitoring.

Molecules that target protein S: Protein S is a cofactor for TFPI and APC which function to restrict thrombin generation. Targeting protein S using a small interfering RNA has been reported to improve hemostasis in hemophilia mice (Prince et al, 2020) and a protein S antibody has been used to enhance FIX replacement therapy in the thrombin generation of patients with hemophilia B (Wilson et al, 2024). Free protein S and total protein S, which also measures protein S complexed with the complement regulator, C4b-binding protein, are routinely available in many tertiary hemostasis laboratories should measurement be required for drug monitoring.

Thrombin generation assays: Thrombin generation (TG) assays are global assays which can assess overall hemostatic potential and highlight hyper- or hypocoagulability in plasma (Ninivaggi et al, 2021). There are several in-house and commercial chromogenic or fluorogenic TG assays available which commonly trigger TG using tissue factor, FXIa, or FIXa. Due to lack of standardization, there is poor correlation between them (Devreese et al, 2007). Despite these issues, assays of TG are often used in pharmaceutical trials to assess the effect of new molecules on hemostasis.

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