



Factor VIII and Factor IX Activity Measurements for Hemophilia Diagnosis and Related Treatments

Annette E. Bowyer, FIBMS, PhD¹ Robert C. Gosselin, CLS²

¹Department of Coagulation, Royal Hallamshire Hospital, Sheffield, United Kingdom

²Hemostasis and Thrombosis Center, University of California, Davis Health System, Sacramento, California

Address for correspondence Robert C. Gosselin, CLS, Hemostasis and Thrombosis Center, University of California, Davis Health System, Sacramento, CA 95817 (e-mail: rcgosselin@outlook.com).

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Abstract

Accurate measurement of clotting factors VIII (FVIII) or IX (FIX) is vital for comprehensive diagnosis and management of patients with hemophilia A or B. The one-stage activated partial thromboplastin time (aPTT)-based clotting assay is the most commonly used method worldwide for testing FVIII or FIX activities. Alternatively, FVIII and FIX chromogenic substrate assays, which assess the activation of factor X, are available in some specialized laboratories. The choice of reagent or methodology can strongly influence the resulting activity. Variation between one-stage FVIII or FIX activities has been reported in the measurement of some standard and extended half-life factor replacement therapies and gene therapy for hemophilia B using different aPTT reagents. Discrepancy between one-stage and chromogenic reagents has been demonstrated in some patients with mild hemophilia A or B, the measurement of some standard and extended half-life factor replacement therapies, and the transgene expression of hemophilia A and B patients who have received gene therapy. Finally, the measurement of bispecific antibody therapy in patients with hemophilia A has highlighted differences between chromogenic assays. It is imperative that hemostasis laboratories evaluate how suitable their routine assays are for the accurate measurement of the various hemophilia treatment therapies.

Keywords

- ▶ one-stage factor assays
- ▶ chromogenic factor assays
- ▶ factor VIII
- ▶ factor IX
- ▶ hemophilia
- ▶ gene therapy

The X-linked, hemostatic disorders of hemophilia are caused by the absence or reduction of clotting factor VIII (hemophilia A, HA) or factor IX (hemophilia B, HB), which can lead to uncontrolled bleeding. It is estimated that more than 300,000 people have hemophilia worldwide.¹ The lower the measurable factor VIII (FVIII:C) or factor IX (FIX:C) functional “coagulant” activity, the more significant the bleeding diathesis. HA and HB are classified into severe (FVIII:C or FIX:C < 1 dL), moderate (FVIII:C or FIX:C 1–5

IU/dL), and mild (FVIII:C or FIX:C > 5 to < 40 IU/dL) disorders based on the level of clotting factor activity.² Patients with mild HA or HB have fewer bleeding problems than those with moderate or severe forms, often only requiring replacement factor therapy following significant trauma or postoperatively. Patients with moderate hemophilia may bleed following minor trauma, whereas severely affected patients may exhibit spontaneous bleeding, which can occur into joint spaces (hemarthroses). Bleeding in untreated severe

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hemophilia patients is variable, with annualized bleed rates (ABR) ranging from 0 to more than 50.³ Treatment of hemophilia may be episodic (on-demand) following a bleed, or prophylactic (to prevent future bleeds) via regular injections of factor concentrate. Prior to the last decade, the treatment of hemophilia was with standard half-life (SHL) plasma-derived (pd) or recombinant (r) FVIII or (r)FIX. More recently, modifications to rFVIII or rFIX molecules have extended the half-life of the products in the circulation by around 1.5-fold for FVIII^{4–7} and up to 5-fold for FIX,^{8–10} while novel rebalancing therapies and gene therapy have greatly expanded the treatment options for hemophilia.^{11–13} The prophylactic dosage of clotting factor concentrates may be regulated by a specific regimen of standard doses or tailored to each individual patient following measurement of the peak level (the FVIII:C or FIX:C immediately after treatment) and trough level (the lowest FVIII:C or FIX:C immediately prior to the next dose). The hemostasis laboratory serves a critical role in the diagnosis and management of HA or HB by providing screening tests (prothrombin time [PT] and activated partial thromboplastin time [aPTT]), specific factor assays (e.g., factor VIII, F8), and/or inhibitor studies (e.g., Bethesda assay). Factor assays can be used for diagnostic purposes (e.g., identifying a congenital or acquired factor deficiency), monitoring purposes (assessing the pharmacokinetics of a factor replacement therapy), assessing product quality control (e.g., FVIII:C in cryoprecipitate), or to assess product potency of factor concentrates.

Historically, the problems associated with these assays in the diagnosis and management of hemophilia have been attributed to the variability of results between assays, usually secondary to test methodology, calibration, and reagent (including factor deficient material) sources.^{14,15} Improvements in factor assay performance within and between laboratories have emerged with advancing technologies (automated analyzers), laboratory performance guidelines such as those from the British Committee for Standardization in Haematology [BCSH], and proficiency testing.¹⁶

While biases still exist between laboratories in FVIII and FIX performance, in-house performance of these tests is usually constrained by operational limitations. These are typically instrument related, such as differences in the lower limit of quantitation (LLOQ) and can impact on determining hemophilia severity.

Clinicians are often unaware of laboratory limitations, and most have a relatively naive knowledge of their laboratory performance in clotting factor or inhibitor assays. Replacement products for treating HA and HB that were human (sometimes porcine) derived provided clotting factor activities as expected when using traditional laboratory methods. The expectation that all replacement therapies could be reliably monitored by any reagent or methodology changed when a B-domain-deleted (BDD) rFVIII (ReFacto, Wyeth Pharmaceutical) was approved by the U.S. Food and Drug Administration (FDA) in 2000. It was demonstrated that a ReFacto-specific calibrator was required to obtain accurate results, regardless of method (one-stage clotting assay [OSA]

or chromogenic substrate assays [CSA]).^{17,18} Since that time, there has been a proliferation of new hemophilia treatment strategies, including modified (polyethylene glycol(PEG)ylated, albumin-fused, FC-fusion) extended half-life (EHL) replacement products or gene therapy (in lieu of factor replacement). Each of these has laboratory challenges in accurately measuring factor activity.^{19,20}

FVIII and FIX Factor Assays: General Laboratory Considerations

Unless the equipment and related reagents are designated for a hemophilia treatment center, it is likely that instrumentation and related reagent selection for clotting factor assays will be predicated on modified PT and aPTT assay testing. With the understanding that FVIII and FIX testing may be used outside the scope of hemophilia assessment, there are certain expectations that should be considered when using these platforms outside the general-purpose use of PT/aPTT screening or drug-monitoring testing, including but not limited to the following:

- Variables associated with instrumentation, calibrator source, calibration type and LLOQ, aPTT reagent, factor-deficient plasma source, and sample diluent (–Table 1). Likely, many of these variables are default protocols embedded within an instrument testing menu. Modifications or alterations of these defaulted protocol(s), including alternative reagents or calibrators, may constitute an in-house or laboratory-developed test which may have regulatory requirements for validation prior to clinical use.
- The selection of whether to use OSA or CSA may be predicated on additional considerations or restrictions such as reagent contracts, instrumentation, accreditation, or regional regulatory requirements.
- Anticoagulant interferences including heparins, parenteral direct thrombin inhibitors (DTIs), and direct oral anticoagulants (DOACs) may interfere with accurate performance of either OSA or CSA methods, with possible underestimation of factor activity. Some CSA assays may be less affected than other assays due to heparin neutralizers in reagents and higher sample dilutions. Some drugs may mimic a factor inhibitor and exhibit assay non-parallelism.²¹
- Nonspecific inhibitors (e.g., lupus anticoagulant) or other drug effects (e.g., lipoglycopeptide antibiotics) may interfere with OSA testing, although the inhibitor effect may be diminished due to sample dilutions for OSA testing.^{22–24} CSA assays may be less affected due to higher sample dilution.
- Porcine-derived products (Obizur, porcine rFVIII, BAX801) are reliably assessed using OSA methods; CSA methods are less reliable.^{25–28}
- For animal samples, OSA may be the preferred (only) option, although animal factor levels may not be comparable to humans, and thus calibration alterations may be required.²⁹

Table 1 General factor assay considerations

Automation	Most coagulation analyzers have the capacity to perform OSA factor assays, usually dedicated to a single source of factor-deficient plasma, calibrator, and controls with clot detection by optical or mechanical means. Few instruments are programmed for CSA factor methods. Alterations to the instrument protocol may be required when using alternative materials and may require additional validation as required by regional regulatory agencies. Analyzers may have a dual OSA platform to accurately assess low factor activity levels (e.g., <15 IU/dL). Instruments without CSA protocols would require programming to adapt the methodology into an automated platform. CSA methods may also require dual platforms, where the low factor activity measurements requiring longer incubation and read times. Automation provides automatic calibration services, calibration curve fitting, patient calculations, and some instruments provide parallelism check to indicate presence of inhibitor
Calibration	All OSA and CSA methods require a calibration to provide a quantitative measurement. Calibrators should be traceable to recognized standard from reputable organizations such as World Health Organization (WHO), National Institute for Biological Standards and Control (NIBSC), or International Society of Thrombosis and Haemostasis (ISTH), and have an assigned activity using either OSA or CSA method. OSA methods typically have 5–7 calibration points, whereas CSA methods may have less, but more than 2 calibration points. Calibration curves can be linear, log–log; linear–log, polynomial, and use of derivatives. Patient samples should be tested at least at three different dilutions for OSA, and single dilution for CSA method
Factor-deficient plasma	Factor-deficient plasma (DP) can be immunodepleted, chemical depleted, or from congenital deficiency sources (with or without VWF in FVIII DP) and must contain <1 IU/dL of factor. DP may be lyophilized or frozen plasma. Whether one source is better than the other is for debate, each with their own advantages and disadvantages (e.g., cost, stability, reuse or freezing, instrument compatible without transferring to secondary vials, VWF levels, etc.)
aPTT reagent	Most commercial aPTT reagents are designed to be sensitive to changes in FVIII and FIX levels. Activator content (silica, kaolin, ellagic acid, polyphenols) and phospholipid type and source (e.g., animal, plant, or synthetic) are confounders to responsiveness of factor testing. Reagent considerations are required when the EHL factor testing is being performed on patients treated with EHL replacement products. Note that use of OSA for QC purposes, especially on cryoprecipitate products, may require a higher predilution of the sample than required with patient samples
Diluent	Suitable diluents used for making calibrator and plasma sample diluents include saline or buffered solutions such as Owren's, Owren-Koller, HEPES, imidazole buffer, FVIII or FIX-deficient plasma, and others

Abbreviations: aPTT, activated partial thromboplastin time; CSA, chromogenic assay; EHL, extended half-life; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid; OSA, one-stage assay; VWF, von Willebrand factor.

FVIII and FIX Measurements for Diagnosis

The separation of hemophilia into HA and HB was first made in 1952³⁰ following the development of the OSA and two-stage clotting assays.^{31–33} A variation of the OSA has been in world-wide use since that time, whereas the two-stage clotting assay is now performed only in a handful of specialized laboratories. The OSA is a modification to the aPTT by dilution of test plasma and addition of plasma that is completely devoid of the clotting factor to be tested (i.e., either FVIII or FIX) for hemophilia diagnosis. This factor-deficient plasma has a prolonged aPTT and any FVIII or FIX present in the patient plasma will shorten (or “correct”) the aPTT. The clotting times generated in the patient plasma are compared with those of a reference or calibrator plasma and the concentration determined via a calibration curve.³⁴ The OSA is a heterogeneous test, with numerous combinations of reagents and instrumentation used worldwide. General considerations for factor assays include the use of automation, calibration, factor-deficient plasma source, aPTT reagent source, number of plasma dilutions, and diluent (► **Table 1**). aPTT reagents contain a contact activator, with a variety of materials used, including ellagic acid, kaolin or silica derivatives, as well as a variety of animal or plant-sourced phospholipids including phosphatidylserine, phos-

phatidylcholine, phosphatidylinositol, phosphatidylethanolamine, or sphingomyelin.³⁵ The sensitivity of aPTT reagents to mild deficiencies of FVIII and FIX is inconsistent.^{36–38} While it is recommended that a prolongation to the aPTT be present when FVIII, FIX, or FXI are less than 30 IU/dL,³⁹ it follows that some reagents may have a normal aPTT in the presence of mild HA or HB which may compromise diagnosis.

In the 1980s, a chromogenic substrate FVIII assay (CSA) was introduced.⁴⁰ This assay involves activation of the test FVIII, then, in combination with added FIXa, activates FX to FXa. The FXa generated cleaves a FXa-specific chromophore and the resultant change in color is measured via optical density (OD). The OD of the test plasma is compared with those of a reference or calibrator plasma and the concentration determined via a calibration curve. The higher the color generation, the greater the level of FVIII. There are several kits on the market, which vary in source of reagents (human, bovine, or a mix), phospholipid type and concentration, buffers, and incubation time.

The chromogenic FIX assay is a recent addition to the laboratory repertoire. The assay principle is also a generation of FXa, then measurement by cleavage of a specific chromogenic substrate. There are, at the time of writing, three chromogenic FIX assay kits available which vary in their constituents and assay conditions.^{41–44}

FVIII and FIX Measurements: Discrepancies in Mild Hemophilia

The diagnosis of severe, moderate, and some mild HA and HB phenotypes can be made by either OSA or CSA. The results of the two methods are generally comparable. However, there are more than 20 mutations in *F8* which are linked to significant discrepancy between OSA and CSA or two-stage clotting assay.³¹ Assay discrepancy was initially reported in 1983 in four patients from two families with a twofold or greater FVIII:C measured by OSA than two-stage clotting assay.⁴⁵ This lower two-stage clotting or CSA form of assay discrepancy has been widely reported in Europe and Australia^{46–50} and is caused by point mutations in *F8* at the A1–A2, A2–A3, or A1–A3 domain interfaces. These have been reported to increase the rate of dissociation of A2 domain leading to premature inactivation of FVIII.^{51–53} The CSA has a longer incubation time than OSA; so, the untimely inactivation of FVIII manifests as reduced CSA activity, whereas the OSA FVIII:C is less impacted. The reverse discrepancy with a lower OSA compared with CSA has also been described. A further 10 mutations in *F8* have been linked to this type of assay discrepancy but in particular one mutation, p.Tyr365Cys/Phe has been commonly described in the United Kingdom.^{54–57} These mutations, along with p.Glu340Lys and p.Ile388Thr, are thought to induce a conformational change resulting in a delay to thrombin activation of FVIII at p.Arg391.^{54,58,59} This causes a lower OSA since FVIII is not activated as quickly as wild-type FVIII, but the longer incubation time in the CSA results in higher or normal activity. The reasons for reverse discrepancy with other mutations that are not near this thrombin activation site are unclear.⁵⁰ Some HA patients with FVIII assay discrepancy have reduced, but still discordant, OSA and CSA; so, the diagnosis is not usually compromised. However, in those patients where one result is within normal limits and the second is reduced, there may be a delay to diagnosis if only one assay method is routinely performed. It is therefore important to measure both OSA and CSA in patients with suspected mild HA. The clinical bleeding phenotype generally coincides with the lowest value observed, but this is dependent on the mutation present.⁵³

Assay discrepancy in HB is rarely described, but when described appears to compromise the severity with patients alternating between moderate and mild HB. One report described 14 of 15 patients with FIX: c.572G > A; p.Arg191His or FIX: c.571C > T; p.Arg191Cys amino acid changes in *F9*. The mean OSA was 0.02 IU/mL and that of CSA was 0.06 IU/mL and patients reported a mild bleeding phenotype.⁶⁰ A further study reported 2.5-fold higher results by OSA compared with CSA.⁶¹ The aPTT reagent composition has also been demonstrated to affect the OSA in patients with mild HB.⁶²

FVIII and FIX Measurements: Monitoring of Standard Half-Life FVIII and FIX

The monitoring of pdFVIII or FIX and most SHL recombinant products are not influenced by OSA reagents. The exception is the B-domain-depleted (BDD) rFVIII concentrate, ReFacto, and its successor ReFacto AF (moroctocog alfa, Pfizer⁶³). FVIII:C levels of ReFacto AF measured by OSA calibrated with a reference plasma may be 20 to 50% lower than expected; however, this difference can be reduced by use of either a product-specific reference plasma (ReFacto laboratory standard, Pfizer) in a standard OSA or using a plasma reference plasma in a CSA.¹⁸ The recovery of ReFacto AF may also depend on the constituents of the aPTT reagent since not all aPTT reagents demonstrate lower results by OSA.^{64,65} Chromogenic FVIII assays also recover close to the expected FVIII:C with SHL products; however, some SHL rFIX products may be underestimated by chromogenic FIX assays.^{66,67} The United Kingdom Haemophilia Centre Doctors' Organization (UKHDCO) has published guidance for the laboratory monitoring of FVIII and FIX concentrates.⁶⁸ A summary of potential differences can be viewed in ►Table 2.

FVIII and FIX Measurements: Monitoring Extended Half-Life FVIII and FIX

One of the disadvantages of treatment with SHL FVIII and FIX therapy is the length of time that the product remains active in the circulation; for FVIII, this is approximately 12 hours

Table 2 Reagent and methodological discrepancy in the measurement of FVIII and FIX molecules

Molecule	One-stage V chromogenic assay discrepancy reported	One-stage assay reagent differences	Chromogenic assay reagent differences	References
Native FVIII	Yes in some patients with mild HA	No	No	48,56
Pd FVIII	Yes	No	No	125
SHL FVIII	Yes with BDD	Yes with BDD	No	65,118,126
EHL FVIII	Yes	Yes	No	68,81,84,127
FVIII mimetics	Yes	Not suitable for use	Yes	100,102,128
Native FIX	Yes in some patients with mild HB	No	No data	60,62,129
Pd FIX	No	Yes but limited data	No	66
SHL FIX	Yes	Yes	Yes	66,90,130
EHL FIX	Yes	Yes	Yes	66,79,90

Abbreviations: BDD, B-domain deleted; EHL, extended half-life; HA, hemophilia A; HB, hemophilia B; Pd, plasma derived; SHL, standard half-life.

Table 3 Modified factor VIII replacement products^{131–135}

Name	Manufacturer	Factor VIII modification	Half-life ^a (h)	Approval date
ELOCTATE/ELOCTA (rFVIII-Fc BDD)	Bioverativ/SOBI	Fusion to Fc domain of IgG1	13–20	FDA Jun 2014 EMA Nov 2015
AFSTYLA (CSL627)	CSL Behring	Single chain–PEGylated	10–14	FDA May 2016 EMA Nov 2015
ADYNOVATE/ADYNOVI (Bax 855)	Takeda (Shire)	20-kDa branched PEGylated	12–15	FDA Dec 2016 EMA Jan 2018
JIVI (BAY 94–9027)	Bayer	Site-specific 60-kDa PEGylated	17–21	FDA Aug 2018 EMA Nov 2018
ESPEROCT (N8-GP)	Novo Nordisk	40-kDa glycoPEGylated	10–14	FDA Feb 2019 EMA Apr 2019

Abbreviations: EMA, European Medicines Agency; FDA, Food and Drug Administration; kDa, kilodalton; PEG, polyethylene glycol.

^aHalf-life represents mean values, and is age dependent (pediatrics vs. adults)—source is prescribing information from each respective drug.

and for FIX approximately 20 hours.⁶⁹ There have been several approaches undertaken to extend the half-life of each protein including fusion with polyethylene glycol (PEG),^{5,6,70–72} albumin,^{73,74} or the Fc fragment of IgG.^{75–77} This has resulted in commercially available EHL FVIII (►Table 3) and FIX (►Table 4) products for the treatment of HA and HB, respectively.

Even with liberal acceptability for factor recovery of 25 to 30%, it became apparent during clinical and laboratory field trials with some EHL products and some aPTT reagents that significant discrepancy in measured FVIII or FIX recovery could be reproducibly demonstrated. It cannot be assumed that EHL molecules that use the same modification technology (such as PEG) will exert the same effect on aPTT reagents that share the same activator and phospholipid source. Likewise, it cannot be assumed that aPTT reagents that share the same activator and phospholipid source will have the same response to those EHL with the same modification.^{78,79} EHL prescribing information may assist clinicians for acceptable or recommended laboratory methods, but it is unlikely that many clinicians are aware of the FVIII or FIX method used in their laboratory and the limitations of those assays. Additionally, most physicians are usually unaware of their laboratory's performance in FVIII, FIX, or related inhibitor assays as assessed by External Quality Assurance programs required for comparing local laboratory performance to regional or international peers.

There are currently five FVIII EHL products available for clinical use (►Table 3). Esperoct (previously N8-GP, Novo

Nordisk, Denmark) was underestimated by up to 50% by silica-activated aPTT SP,^{80,81} while Jivi (previously Bay 94–9027-, Bayer AG, Germany) was underestimated by some silica-based aPTT reagents and overestimated by some kaolin-based aPTT reagents.⁸² The single-chain recombinant FVIII, Afstyla (CSL Behring, Germany), is underestimated by approximately 45% by OSA,^{83,84} and consequently the manufacturer recommends the CSA.⁸⁵ The manufacturer also states that if the OSA is used, then a correction factor of 2 can be applied to obtain the final result. This approach is not internationally recommended due to differences between one-stage assays.⁶⁸ The CSA has been reported to achieve acceptable recovery with all currently licensed EHL FVIII concentrates. Additional modifications to rFVIII, which combines BDD rFVIII with Xten polypeptides and a fragment of von Willebrand factor, have improved the half-life extension to more than threefold compared with SHL in clinical studies.⁸⁶

There are currently three EHL FIX concentrates licensed for use in some countries. These have been modified by Fc fusion,⁷⁷ albumin fusion,⁹ or glycopegylation⁸⁷ (►Table 4). Under or over estimation with aPTT reagents in the OSA has been reported for each product.^{66,79,88–90} CSAs are also varied in their response.^{66,91}

In 2020, there were three guidance publications that provided recommendations for or against FVIII and FIX OSA or CS assays for EHL products.^{68,92,93} Of particular concern is (1) limited data for nondescribed aPTT reagents, (2) insufficient or discordant data obtained from clinical or

Table 4 Modified factor IX replacement products^{136–138}

Name	Manufacturer	Factor VIII modification	Half-life ^a (h)	Approval date
ALPROLIX (rFIX-Fc)	Bioverativ/SOBI	Fusion to Fc domain of IgG1	68–94	FDA Mar 2014 EMA May 2016
IDELVION (rIX-FS CSL654)	CSL Behring	Fusion to albumin	~90	FDA Mar 2016 EMA May 2016
REBINYN/REFIXIA (N9 = GP)	Novo Nordisk	40-kDa glycoPEGylated	70–89	FDA May 2017 EMA Mar 2017

Abbreviations: EMA, European Medicines Agency; FDA, Food and Drug Administration; kDa, kilodalton; PEG, polyethylene glycol.

^aSingle-dose, half-life mean values, and is age dependent (pediatrics vs. adults)—source is prescribing information from each respective drug.

APTT Reagent	Activator	PL source	Eloctate/Elocta			Adynovate/Adynovi			Afstyla			Jivi			Esperoct			Alprolix			Idelvion			Reflexia/Rebinyn		
			rFVIII-Fc			BAX-855			rFVIII-SC			BAY94-9027			N8-GP			rFIX-Fc			rFIX-FP (CSL654)			N9-GP		
			1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
STA PTT Automate	Silica	Cephalin	Green	Green	Green	Green	Green	Green	White	White	White	Yellow	Red	Red	Green	Green	Green	Yellow	Yellow	Yellow	Green	Green	Green	Red	Red	Red
CK Prest	Kaolin	RBT	Green	Green	Green	Green	Green	Green	White	White	White	Yellow	Red	Red	Green	Green	Green	Yellow	Yellow	Yellow	Green	Green	Green	Red	Red	Red
TriniCLOT Auto	Micro-Silica	RBT	Green	Green	Green	Green	Green	Green	White	White	White	Yellow	Red	Red	Green	Green	Green	Yellow	Yellow	Yellow	Green	Green	Green	Red	Red	Red
TriniCLOT HS	Micro-Silica	Pig/Chick phosphatides	Green	Green	Green	Green	Green	Green	White	White	White	Yellow	Red	Red	Green	Green	Green	Yellow	Yellow	Yellow	Green	Green	Green	Red	Red	Red
Cephascreen	Polyphenol	RBT	Green	Green	Green	Green	Green	Green	White	White	White	Yellow	Red	Red	Green	Green	Green	Yellow	Yellow	Yellow	Green	Green	Green	Red	Red	Red
SynathSL	Colloidal silica	Syrthetic	Green	Green	Green	Green	Green	Green	White	White	White	Yellow	Red	Red	Green	Green	Green	Yellow	Yellow	Yellow	Green	Green	Green	Red	Red	Red
SynthaFax	Ellagic acid	Syrthetic	Green	Green	Green	Green	Green	Green	White	White	White	Yellow	Red	Red	Green	Green	Green	Yellow	Yellow	Yellow	Green	Green	Green	Red	Red	Red
APTT-SP	Colloidal silica	Syrthetic	Green	Green	Green	Green	Green	Green	White	White	White	Yellow	Red	Red	Green	Green	Green	Yellow	Yellow	Yellow	Green	Green	Green	Red	Red	Red
Actin	Ellagic acid	RBT	Green	Green	Green	Green	Green	Green	White	White	White	Yellow	Red	Red	Green	Green	Green	Yellow	Yellow	Yellow	Green	Green	Green	Red	Red	Red
Actin FS	Ellagic acid	Soy phosphatides	Green	Green	Green	Green	Green	Green	White	White	White	Yellow	Red	Red	Green	Green	Green	Yellow	Yellow	Yellow	Green	Green	Green	Red	Red	Red
Actin FSL	Ellagic acid	Soy + RBT	Green	Green	Green	Green	Green	Green	White	White	White	Yellow	Red	Red	Green	Green	Green	Yellow	Yellow	Yellow	Green	Green	Green	Red	Red	Red
Pathromtin SL	Silicon dioxide	Plant	Green	Green	Green	Green	Green	Green	White	White	White	Yellow	Red	Red	Green	Green	Green	Yellow	Yellow	Yellow	Green	Green	Green	Red	Red	Red

Fig. 1 Recommended or rejected OSA methods for measuring FVIII and FIX EHL products from recent publications.^{68,92,93} Columns labeled “1” reflect recommendations from Peyvandi et al,⁹² columns labeled “2” from Gray et al,⁶⁸ and columns labeled “3” from Jeanpierre et al.⁹³ Green cells indicate a recommended method, yellow cells indicate insufficient or conflicting clinical or field trial data, and red cells indicate rejected method. A white cell indicates this method was not specifically addressed by authors. Note: Afstyla can only be measured using a suitable FVIII chromogenic assay. Note: For Adynovate/Adynovi, the Kihlberg et al⁶⁰ group could not recommend any OSA method. PL, phospholipid; RBT, rabbit brain thromboplastin.

field studies, and (3) discordant OSA or CSA recommendations between these publications. It is likely that a clinical laboratory will provide a single OSA method for both FVIII and FIX, usually due to contractual agreements or instrument default methods. Therefore, when evaluating the three guidance documents, it is necessary to recommend which OSA method is suitable for monitoring FVIII and FIX EHL concentrates (→ Fig. 1). For CSA methods, there was mostly concordance between these guidance recommendations, with notable exception for Esperoct and Idelvion (→ Fig. 2).

It is imperative that laboratories assess their local assays for suitability to measure EHL FVIII and FIX concentrates that may be used to treat their patients. For those aPTT methods

not adequately described or recommended, implementing a reagent for EHL based on other reagent platforms with similar activator may not be a suitable means for predicting suitability for EHL monitoring (→ Fig. 3). Given the variability within a given reagent platform (→ Figs. 1 and 2), it is likely that more than one FVIII or FIX method may be required to accurately monitor all EHL replacement therapies.

FVIII Measurements: FVIII Mimetics

FVIII mimetics, including emicizumab (Hemlibra, Roche Chugai) and Mim8 (Novo Nordisk), are humanized bispecific antibodies directed to human FIX (FIXa) and FX, which

Method	Eloctate/Elocta			Adynovate/Adynovi			Afstyla			Jivi			Esperoct			Alprolix			Idelvion			Reflexia/Rebinyn		
	rFVIII-Fc			BAX-855			rFVIII-SC			BAY94-9027			N8-GP			rFIX-Fc			rFIX-FP (CSL654)			N9-GP		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Chromogenic VIII Assay ^S	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green
Biophen FVIII	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green
Technochrome FVIII	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green
Coatest FVIII	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green
FVIII Chromogenic	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green
Electrachrome FVIII	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green
Coamatic FVIII	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green
Biophen FIX	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green
Rox FIX	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green

Fig. 2 Recommended or rejected CS methods for measuring FVIII and FIX EHL products from recent publications.^{68,92,93} Columns labeled “1” reflect recommendations from Peyvandi et al,⁹² columns labeled “2” from Gray et al,⁶⁸ and columns labeled “3” from Jeanpierre et al.⁹³ Green cells indicate a recommended method, yellow cells indicate insufficient or conflicting clinical or field trial data, and red cells indicate rejected method. A white cell indicates this method was not specifically addressed by authors.^SGeneric recommendations for CS VIII methods, no specific reagent(s) may be noted. Note: Kihlberg et al⁶⁰ group did not provide recommendations for Adynovate/Adynovi based on limited clinical or field trial data.

APTT Reagent	Activator	PL source	Eloctate/Elocta			Adynovate/Adynovi			Jivi			Esperoct			Alprolix			Idelvion			Reflexia/Rebinyln		
			rFVIII-Fc			BAX-855			BAY94-9027			N8-GP			rFIX-Fc			rFIX-FP (CSL654)			N9-GP		
			1	2	3	1	3		1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
STA PTT Automate	Silica	Cephalin	Green	Green	Green	Green	Green	Green	Red	Red	Red	Red	Red	Red	Green	Green	Green	Red	Red	Red	Red	Red	Red
TriniCLOT Auto	Micro-Silica	RBT	Green	Green	Green	Green	Green	Green	Yellow	Green	Green	Red	Red	Red	Green	Green	Green	Red	Red	Red	Red	Red	Red
TriniCLOT HS	Micro-Silica	Pig/Chick phosphatides	Green	Green	Green	Green	Green	Green	Yellow	Green	Green	Red	Red	Red	Green	Green	Green	Red	Red	Red	Red	Red	Red
Synathsil	Colloidal silica	Synthetic	Green	Green	Green	Green	Green	Green	Yellow	Green	Green	Red	Red	Red	Green	Green	Green	Red	Red	Red	Red	Red	Red
APTT-SP	Colloidal silica	Synthetic	Yellow	Green	Green	Green	Green	Green	Red	Red	Red	Red	Red	Red	Yellow	Green	Green	Red	Red	Red	Red	Red	Red
Pathromtin SL	Silicon dioxide	Plant	Green	Green	Green	Green	Green	Green	Red	Red	Red	Red	Red	Red	Green	Green	Green	Red	Red	Red	Red	Red	Red
Cephascreen	Polyphenol	RBT	Green	Green	Green	Green	Green	Green	Green	Green	Green	Red	Red	Red	Green	Green	Green	Red	Red	Red	Red	Red	Red
SynthaFax	Ellagic acid	Synthetic	Yellow	Green	Green	Green	Green	Green	Red	Red	Red	Red	Red	Red	Green	Green	Green	Red	Red	Red	Red	Red	Red
Actin	Ellagic acid	RBT	Green	Green	Green	Green	Green	Green	Yellow	Green	Green	Red	Red	Red	Green	Green	Green	Red	Red	Red	Red	Red	Red
Actin FS	Ellagic acid	Soy phosphatides	Green	Green	Green	Green	Green	Green	Red	Red	Red	Red	Red	Red	Yellow	Green	Green	Red	Red	Red	Red	Red	Red
Actin FSL	Ellagic acid	Soy + RBT	Green	Green	Green	Green	Green	Green	Red	Red	Red	Red	Red	Red	Yellow	Green	Green	Red	Red	Red	Red	Red	Red

Fig. 3 Aligning aPTT reagents by activators (silica or polyphenols) of the recommended or rejected CS methods for measuring FVIII and FIX EHL products from recent publications.^{68,92,93} Columns labeled “1” reflect recommendations from Peyvandi et al,⁹² columns labeled “2” from Gray et al,⁶⁸ and columns labeled “3” from Jeanpierre et al.⁹³ Green cells indicate a recommended method, yellow cells indicate insufficient or conflicting clinical or field trial data, and red cells indicate rejected method. A white cell indicates this method was not specifically addressed by authors. PL, phospholipid; RBT, rabbit brain thromboplastin.

activate FX in the absence of FVIIIa.^{94,95} Emicizumab is licensed for use in Europe, Australia, and the United States, in patients with HA and anti-FVIII antibodies and severe HA without antibodies.⁹⁶ Bispecific antibodies do not require preactivation to be functional, unlike FVIII; thus, the action on FX is more rapid⁹⁷ and this impacts on some hemostasis tests.^{98–100} The aPTT dramatically shortens, even at subtherapeutic concentrations and any aPTT-based assays will also be affected, including OSA for FVIII.^{98,101} OSA FVIII is artificially increased, often far above the top of the reference range. Several organizations have published guidance for the monitoring of patients receiving emicizumab therapy.^{102,103} Quantitative measurement of the emicizumab drug concentration can be made by modifying the OSA to use an emicizumab-specific calibrator and high plasma dilutions. The use of CSA which use bovine FX can measure endogenous FVIII due to their insensitivity to emicizumab at therapeutic concentrations.¹⁰⁴ The monitoring for FVIII inhibitors in patients receiving these products create an additional challenge, with modification to existing Bethesda or Nijmegen methods having been described.¹⁰⁵

FVIII and FIX Measurements: Rebalancing Therapies

New classes of drugs, not based on the replacement of FVIII or IX molecules, are currently in clinical trials for prophylaxis of hemophilia patients. They include the small interference RNA (siRNA) antithrombin knockdown therapy (fitusiran, Sanofi),¹⁰⁶ anti-tissue factor pathway inhibitor (TFPI) monoclonal antibodies (concizumab, Novo Nordisk, and marstacimab, Pfizer),^{107,108} and serpins against activated protein C.¹⁰⁹ These drugs are able to increase thrombin generation in the absence of FVIII and FIX, although it is unlikely that monitoring these rebalancing therapies using routine hemostasis assays will be clinically useful, or easily applied.

FVIII and FIX Measurements: Gene Therapy

Gene therapy trials have been running for FVIII and FIX for several years, with numerous currently active and recruiting clinical trials for both HA and HB.^{12,110–112} Recent guidance from the U.S. FDA for human gene therapy in hemophilia has a section detailing the necessity for laboratory testing to include both chromogenic and one-stage assays with a variety of reagents in vitro and a comparative field study in patient plasma.¹¹³

Differences in FVIII or FIX transgene expression measured by OSA and CSA have been reported in hemophilia patients who have received certain gene therapy products (–Table 5). In HA, an approximate 1.6-fold higher FVIII:C OSA FVIII:C than CSA has been reported.^{114–117} Curiously, this is the reverse of the pattern observed in some BDD-rFVIII molecules.^{17,118} Rosen et al proposed that the higher OSA compared with CSA in AAV5-FVIII-SQ molecules is caused by accelerated early FXa and thrombin generation, and shorter clotting times generate higher reported FVIII activity levels. This trend was recorded in transgene FVIII:C and the rFVIII-SQ molecule.¹¹⁹ Kinetic assays highlighted the assay differences, but the underlying mechanism is currently unknown.

There has been a difference in recovery of FVIII:C with a limited number of different aPTT reagents in the clinical trials. Ideally, laboratory field studies would provide performance characteristics for OSA or CS methods not used in the clinical trials.¹¹⁹

Discrepancy between OSA and CSA FIX:C has also been reported following FIX gene therapy for HB. Gene therapy approaches that use FIX-Padua, a naturally occurring mutation with higher FIX:C than wild-type FIX,¹²⁰ have observed differences in one-stage FIX:C measured using a variety of aPTT reagents and also between OSA and CSA in both the transgene FIX expressed by patients and plasma spiked with the FIX molecule.^{121–124} The degree of OSA versus CSA discrepancy may be up to twofold; again, OSA activities

Table 5 Selected OSA methods from clinical trials for FVIII and FIX gene therapy

	OSA/CA (slope) Hemophilia A ¹¹⁹	OSA/CA (ratio) Hemophilia B ¹²¹	OSA/CA (slope) Hemophilia B ^a
Actin FS	1.29–1.88	ND	2.03
Actin FSL	1.52–1.66	1.2–1.6	1.10
SynthasIL	1.53–2.01	1.3–2.4	1.38
Triniclot aPTT HS	1.67	ND	ND
CK Prest	ND	0.7–2.2	ND
PTT-Automate	ND	1.0–2.8	ND
Pathromtin SL	ND	ND	1.49

Abbreviations: CA, chromogenic assay; ND, no data; OSA, one-stage clotting assay.

^aAuthor (RCG) unpublished data.

are higher than CSA, but the degree of discrepancy between OSA and CSA seems to vary between patients.¹²¹

With noted observations of differences between OSA and CS methods in clinical studies, the primary endpoint in hemophilia gene therapy is the phenotype of the patient. It is unclear whether routine monitoring is required for gene therapy but is likely necessary for patients who require surgical intervention. It is unknown whether additional factor replacement therapies would be required in gene therapy patients with acute bleeding events, which may add an additional confounder to interpreting OSA or CS results. Hemostasis laboratories may be required to establish unfamiliar assays to facilitate consistent management of gene therapy patients within their center and for those who travel between centers.

Conclusions

For decades, the clinical laboratory has provided the necessary testing for the diagnosis and management of patients with hemophilia. The OSA FVIII and FIX methods are still the traditional workhorses in assessing these patients, with improvements in testing accuracy and precision resulting from technological advances, guidance documents, and robust external proficiency assessment programs. However, the diagnostic limitations of OSA in certain gene mutations and the OSA challenges associated with monitoring modified factor replacement or gene therapies require clinical laboratories to consider secondary options for FVIII and FIX testing to aide clinicians who manage hemophilia patients. A multidisciplinary approach between clinical and laboratory teams is necessary to provide optimum diagnosis and monitoring of treatment for patients with hemophilia. A combination of OSA and chromogenic assay for both FVIII and FIX would appear to be the most favorable test combination to address the current diagnostic and monitoring challenges in hemophilia patients.

Conflict of Interest

A.E.B. reports grants and personal fees from Novo Nordisk, personal fees from Stago, personal fees from Takeda, grants and personal fees from Roche, personal fees from

Sobi, and personal fees from Pfizer, outside the submitted work.

R.C.G. reports personal fees from Sysmex America Incorporated and from Diagnostica Grifols, and honoraria from Diagnostica Stago, outside the submitted work.

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