

Laboratory measurement of factor replacement therapies in the treatment of congenital haemophilia: A United Kingdom Haemophilia Centre Doctors' Organisation guideline

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Abstract

Assay discrepancies can occur with laboratory monitoring of FVIII and FIX replacement therapy, particularly for the extended half-life products. This guideline collates current published data and provides advice on appropriate choice of assays for laboratory measurement of replacement therapy for patients with Haemophilia A and B without inhibitors. It is recommended that each haemophilia centre should ensure that appropriate laboratory assays are available for FVIII and FIX products in local clinical use. Patient samples should be assayed against calibrators traceable to WHO Plasma International Standards. Assay discrepancies are common especially for the extended half-life FVIII and FIX products, and assays of these products may need to be verified with the specific CFC being used.

KEYWORDS

chromogenic assay, extended half-life, factor concentrate, haemophilia, laboratory monitoring

1 | INTRODUCTION

Laboratory monitoring of factor (F) VIII or FIX replacement therapy for treatment of haemophilia A or B is performed to ensure optimal therapy. A wide variety of commercially available one-stage clotting assays or chromogenic assays of FVIII and FIX is used for this purpose. Discrepancies between results obtained using the different assays have been described in hereditary haemophilia A^{1,2} and haemophilia B,^{3,4} in the absence of replacement therapy, and

after gene therapy for both haemophilia A and B.^{5,6} Although discrepancies after infusion of FVIII have been recognized since the advent of recombinant FVIII over 20 years ago,^{7,8} this guideline will highlight recent studies of enhanced half-life FVIII and FIX products which demonstrate discrepancies in results both between chromogenic and one-stage clotting assays, and within assay type. There is currently no published consensus or guidance on what magnitude of discrepancy after concentrate infusion is acceptable for clinical use, and this may vary according to the factor level and the clinical



setting. Some experts have considered a difference of up to 20% in the measured level from the calculated factor treatment level, based on the labelled potency to be clinically acceptable⁹ whereas a number of laboratory assay studies have used 25% or 30%.¹⁰ If one assay consistently gives results 20% below the target based on potency whereas another is 20% above, then both may be suitable despite the 40% difference between them.

The unitages of clotting factor concentrates (CFC) as stated on the potency label are relative to the WHO concentrate International Standards for FVIII or FIX and are used for patient dosing in clinical trials to establish clinical efficacy. WHO plasma International Standards or laboratory standards or calibrators traceable to the plasma International Standards are routinely employed for measurement of factor levels in patient plasmas. Laboratory assays which recover values close to the calculated treatment level based on the potency label are therefore acceptable for use in monitoring that product. Assays which underestimate factor levels relative to the potency could lead to overtreatment if the dose is adjusted to achieve specific target levels, thus increasing cost of care. Conversely, adjusting dosing based on an assay which overestimates relative to the potency label could lead to under dosing of the patient with risk of bleeding and apparent decreased in efficacy.

A number of published studies assessing relationships between results obtained using different assays have used samples constructed *in vitro* by addition of CFC to factor deficient plasma. These are described as 'spiked' or 'mock' samples and evidence to date for Elocta¹¹ and Refixia¹² suggest that spiked samples are commutable. These spiked samples are helpful for identification of reagent and instrument platform differences. However, it is possible that such samples behave differently to samples obtained postinfusion of the same factor concentrate from haemophilia patients, due to *in vivo* processing or interactions. For this reason, findings based on spiked samples should be verified using samples collected from patients after infusion of CFCs (postinfusion) following local verification procedure or recommendation from guidelines such as ISO 15189¹³ and CLSI guidelines.^{14,15} A number of products containing FVIII or FIX which have been modified to extend the half-life are licensed or in development. This guidance document will address the laboratory monitoring of currently available standard half-life products and modified FVIII or FIX CFCs for which there are sufficient data available to make some recommendations. These products and some of their characteristics are summarized in Tables 1 and 2. Further information on reagents described within this guideline is summarized in Table S1.

2 | METHODOLOGY

Publications known to the writing group were supplemented with additional papers identified by searching Medline/PubMed for relevant field studies and by screening reference lists. The European Medicines Agency Summary of Product Characteristics were reviewed for each factor concentrate. For clotting factor concentrates

(CFC) with limited published and/or controversial data, the authors recommend laboratories to undertake local verification of their routine assays prior to testing patient samples. The writing group produced the draft guideline which was subsequently reviewed by the UKHCDO Advisory committee and the British Society for Haematology (BSH) Thrombosis and Haemostasis Task Force. The 'GRADE' system does not apply (because clinical trials are not available to support the 'best practice' recommendations that have been generated) and was therefore not used.

3 | GENERAL OVERVIEW

Results of one-stage FVIII or FIX clotting factor assays can be influenced by the APTT reagent, calibrator, deficient plasma, analyser, calibration process and assay design. Sample handling and assay design should follow current British Committee for Standards in Haematology (BSH) guidance.¹⁶ The APTT reagent has been identified as the most important cause of assay variability for some products as discussed in this guideline, and changing any of these assay components has the potential to alter the results obtained. In addition, batch variability of an APTT reagent should also be considered. Results of different chromogenic assays can be influenced by use of different kit and reagents and by how these are applied locally. Full details of assays are not always included in publications, and it is the opinion of the authors that they should be.

Laboratories should use an assay which has been locally verified as suitable for use with the specific CFC being used, even when different CFCs have similar modifications. For example, B-domain-deleted FVIII and pegylated B-domain-deleted FVIII from different manufacturers may behave differently with the same reagents. Communication with pharmacies and haemophilia centres on products currently available may also help to identify assay requirement for laboratories.

The relationship between results obtained by assays may vary according to the levels of FVIII or FIX. To address this, data are needed for samples containing low (<10 IU/dL), medium (20-50 IU/dL) and high (60-120 IU/dL) levels of FVIII or FIX to support recommendations on assay selection. In the absence of evidence to the contrary, blood samples should be collected and processed in the same way for all FVIII or FIX assays used for diagnosis and treatment monitoring. Blood should be collected into citrate tubes containing 0.105-0.109 mol/L trisodium citrate maintaining the proportion 9:1. For FVIII/FIX assays, samples should be tested within 4 hours of collection except where there are data to support longer intervals between sample collection and processing/testing.¹⁶

Recommendations

1. Each haemophilia centre should ensure that appropriate laboratory assays are available for FVIII and FIX products in local clinical use.



**TABLE 1** FVIII products licensed and available in the UK

Product type	Brand name	INN	Manufacturer	Reagents found suitable	Reagents to avoid
Plasma derived	Optivate		Bio Products Laboratory	NSR	NSR
	Haemoctin		Biotest	NSR	NSR
	Beriate		CSL Behring	NSR	NSR
	Monoclote-P		CSL Behring	NSR	NSR
	Emoclot		Kedrion	NSR	NSR
	Fanhdi		Grifols	NSR	NSR
Recombinant	Advate	Octocog alfa	Shire	NSR	NSR
BDD FVIII	Refacto AF	Moroctocog alfa	Pfizer	NSR	NSR
	NovoEight	Turoctocog alfa	Novo Nordisk	NSR	NSR
	Nuwiq	Simoctocog alfa	Octapharma	NSR	NSR
Extended half-life	Elocta	Efmoroctocog alfa	Swedish Orphan Biovitrum	Actin FS, Actin FSL, Auto APTT, Cephascreeen, CK Prest, Pathromtin SL, SynthASil, Chromogenic assays	NSR
	N8-GP ^a	Turoctocog alfa pegol	Novo Nordisk	Actin, Actin FS, Pathromtin SL, SynthAFax, DG Synth, chromogenic assays	APTT-SP
	Jivi	Damoctocog alfa pegol	Bayer AG	Actin FSL, Pathromtin SL, SynthASil, Chromogenic assays	Actin FS, CK Prest, APTT-S, PTT-A
	Afstyla	Lonoctocog alfa	CSL Behring	Chromogenic assays only	One-stage assays
	Adynovi	Rurioctocog alfa pegol	Shire	NSR	NSR

Abbreviations: INN, International Nonproprietary Names; NSR, no specific recommendation.

^aCurrently licensed in the US.

- Because of the need for assays tailored to the CFC used, it is essential that every request for a FVIII or FIX level for monitoring of replacement therapy clearly identifies which CFC has been used for treatment of the patient and details the time of sampling relative to timing of CFC infusion.
- We recommend laboratories use an assay which has been verified for use with the specific CFC being used, with calibrators traceable to WHO International Standards.
- We recommend using assays which have been studied at low, medium and high levels and give results within 20% of target based on potency label in samples with >30 IU/dL activity, and within 30% for samples with 10-30 IU/dL. It is generally uninformative to use % differences for samples with levels <10 IU/dL.
- If an assay has not been studied at all three levels, we recommend that more data are generated to inform decision making before such an assay can be considered suitable for full routine use in clinical practice.
- We recommend that laboratories should participate in an external accredited proficiency testing programme which includes testing of postinfusion samples, ideally covering all factor replacement products that may require monitoring in that laboratory.

4 | FACTOR VIII PRODUCTS

In Europe, in accordance with the European Pharmacopoeia monograph for FVIII,¹⁷ potency labelling for all current licensed FVIII products is by chromogenic assay against the current International Standard, the 8th International Standard for Blood Coagulation Factor VIII, 07/350. The pharmacopoeia monograph specifies that the measured potency should be within 80%-120% of the stated potency. A number of CE marked chromogenic assay kits are available for measurement of FVIII activity. Table 1 lists some FVIII products licensed and available in the UK.

4.1 | Standard half-life FVIII concentrates

4.1.1 | Plasma-derived FVIII

One-stage and chromogenic FVIII assays which have been verified for use in diagnosis of haemophilia A are suitable for monitoring replacement with plasma-derived FVIII without need for product specific calibrators. In postinfusion samples, differences between chromogenic and one-stage assays calibrated with plasma standards were <15% for Hemofil M, Emoclot and Fanhdi.⁹ Differences between chromogenic



TABLE 2 FIX products licensed and available in the UK

Product type	Brand name	INN	Manufacturer	Reagents found suitable	Reagents to avoid
Plasma derived	Replenine-VF		Bio Products Laboratory	NSR	NSR
	Haemonine		Biotest	NSR	NSR
	Beriplex		CSL Behring	NSR	NSR
	Octaplex		Octapharma	NSR	NSR
Recombinant	BeneFIX	Nonacog alfa	Pfizer	NSR	NSR
	Rixubis	Nonacog gamma	Shire	NSR	NSR
Extended half-life	Alprolix	Eftrenonacog alfa	Swedish Orphan Biovitrum	Actin, Actin FS, Actin FSL, Pathromtin SL, Cephascreen, SynthASil	CK Prest and may be other Kaolin-based reagent
	Idelvion	Albutrepenonacog alfa	CSL Behring	Pathromtin SL, SynthASil	CK Prest, SynthAFax Actin FS
	Refixia	Nonacog beta pegol	Novo Nordisk	SynthAFax, DG Synth, Cephascreen	APTT-SP, Actin, Actin FS, Actin FSL, SynthASil, Pathromtin SL

Abbreviations: INN: International Nonproprietary Names; NSR: no specific recommendation.

and one-stage clotting assays observed in a multicentre proficiency testing exercise for a postinfusion sample containing Haemate were also not sufficiently discrepant to influence patient management.¹⁸

Recommendation for monitoring plasma-derived FVIII

1. We recommend that one-stage clotting or chromogenic FVIII assays calibrated with plasma standards, traceable to WHO International Standard for Plasma FVIII can be used for monitoring plasma-derived FVIII concentrate infusions, unless there is specific evidence to the contrary.

4.1.2 | Full-length recombinant FVIII

It is recognized that results of chromogenic FVIII assays may be 20%-40% higher than results obtained by one-stage clotting assay.^{7,19} Recent data on measurement of postinfusion samples containing Advate, using factor deficient plasma containing VWF in multicentre proficiency testing exercises, have indicated that differences between one-stage and chromogenic assays were <8% on average.^{11,20} Of note, in 2011, a study of measurement of postinfusion samples containing Advate showed a difference of ~40% between results obtained with SynthASil/IL FVIII deficient plasma/IL calibrant/IL analysers and Actin FS/Siemens FVIII deficient/Siemens calibrator/Symex analysers²⁰; however, a repeat study in 2016 showed a difference of only 2%, indicating that relationships between results with different methods are not always constant over time.

Recommendation for monitoring full-length recombinant FVIII

1. We recommend that one-stage clotting and chromogenic FVIII assays calibrated using plasma standards can be used for

monitoring full-length recombinant FVIII concentrate infusions, unless there is method specific evidence to the contrary.

4.1.3 | B-domain-deleted FVIII

ReFacto AF

ReFacto AF is a B-domain-deleted human coagulation factor VIII with 1438 amino acids. It has similar post-translational modifications to those of the plasma-derived molecule.

The manufacturer's Summary of Product Characteristics (SPC)²¹ recommends using chromogenic assay for monitoring patients' factor VIII activity levels during treatment with ReFacto AF. The SPC also stated that FVIII activity by one-stage clotting assay can be significantly affected by both the type of APTT reagent and the reference standard used in the assay. Lower assay results (20%-50%) can be expected by some one-stage clotting assays in comparison with chromogenic assays. The ReFacto AF laboratory standard can be used as a calibrator to correct for this discrepancy.

A number of studies utilizing both spiked and postinfusion samples have identified higher results with chromogenic assays compared to some one stage by up to ~50% when one-stage assays are calibrated with plasma standards.^{8,20,22,23} As the chromogenic assay is used for potency assignment, the higher result is more likely to reflect true FVIII recovery. In one study, a product specific calibrator was required to deliver agreement with chromogenic assay if Actin FS/Siemens reagents were used for one-stage assay but not if SynthASil/IL reagents were used.²⁰

Recommendations for monitoring ReFacto AF

1. We recommend that chromogenic FVIII assays calibrated with a plasma standard are used for monitoring ReFacto AF.



2. One-stage clotting FVIII assays are suitable for monitoring ReFacto AF provided they are calibrated with product specific calibrator, unless there is method specific evidence that the one-stage assay calibrated with plasma standard is in agreement with chromogenic results.

NovoEight

NovoEight is a B-domain-truncated recombinant human coagulation factor VIII (B-domain consists of 21 amino acids of the wild-type B-domain) without any other modifications in the amino acid sequence. This B-domain-truncated human coagulation factor VIII has 1445 amino acids with an approximate molecular mass of 166 kDa. The SPC states that factor VIII activity results by one-stage clotting assays can be significantly affected by the APTT reagent and the reference standard used and that there are significant discrepancies between assay results obtained by one-stage clotting assay and the chromogenic assay.²⁴

A field study of spiked plasma involved 36 laboratories in 19 countries; four concentrations of NovoEight were measured: 3, 20, 60 and 90 IU/dL. Chromogenic assay measured close to target at the trough level but overestimated at a level of 90 IU/dL by 20%. The one-stage clotting assay recovery was 150% of target at 3 IU/dL, 120% of target at 20 IU/dL reducing to 92% of target at 90 IU/dL. The manufacturers concluded that the use of a product-specific standard was not necessary for NovoEight.²⁵ These differences fall within our recommended tolerance for acceptable variation of assay measurement (see general recommendations).

Recommendations for monitoring NovoEight

1. We recommend that one-stage or chromogenic FVIII assays calibrated with plasma standards can be used for monitoring NovoEight, unless there is method specific evidence to the contrary.

Nuwiq

Nuwiq is a B-domain-deleted recombinant FVIII with a 16 amino acid linker. It has 1440 amino acids with a sequence comparable to the B-domain-deleted 90 + 80 kDa form of human plasma factor VIII. The SPC states that factor VIII activity results by one-stage clotting assays can be significantly affected by the APTT reagent and the reference standard used and that there are significant discrepancies between assay results obtained by one-stage clotting assay and the chromogenic assay.²⁶

Published results of FVIII assay were ~15% higher by chromogenic assay compared to one-stage clotting assay (method details not stated) in postinfusion samples collected from adults²⁷ and children.²⁸ A field study of spiked plasma involved 49 laboratories in nine countries; four concentrations of Nuwiq were measured: 1, 5, 30 and 100 IU/dL. Chromogenic assays were on average up to 17% higher than mean one stage. There were only minor differences between results obtained with different reagents. The authors concluded that Nuwiq can be measured with both one-stage and chromogenic assays without the need for a product specific standard.²⁹

Recommendations for monitoring Nuwiq

1. We recommend that one-stage and chromogenic FVIII assays calibrated with plasma standards can be used for monitoring Nuwiq, unless there is method specific evidence to the contrary.

4.2 | Modified extended half-life FVIII molecules

Elocta

Elocta is a recombinant B-domain-deleted factor VIII Fc fusion protein (rFVIII-Fc) with 1890 amino acids covalently linked to the Fc domain of human immunoglobulin G1. The SPC states that factor VIII activity results by one-stage clotting assays can be significantly affected by the APTT reagent and the reference standard used. There is no specific information on one-stage clotting assay and chromogenic assay discrepancies.³⁰

Elocta Factor VIII activity (by one-stage clotting and chromogenic assays) was stable in postinfusion citrated whole blood stored at 24 or 33°C for at least 8 hours prior to analysis.³¹

A field study has been published in which 30 laboratories in seven countries assayed haemophilic plasma samples spiked with rFVIII-Fc at 5, 22 or 87 IU/dL.³² Results of chromogenic assays in the presence of rFVIII-Fc were approximately 20% higher than one-stage assay results. Results of one-stage clotting assays in samples containing Advate or Elocta were within 10% of the expected recovery for samples with 20 or 80-90 IU/dL, with 15%-20% overestimation (approximately 5 IU/dL absolute difference) for both products studied. A clinical study³³ using Actin FSL for one-stage and Biophen chromogenic demonstrated that the relationship between the one-stage clotting and chromogenic assays was consistent in 185 postinfusion samples with concentrations between 0 and 250 IU/dL FVIII (correlation $r = 0.95$).

Recommendations for monitoring Elocta

1. APTT reagents which can be used in a one-stage clotting FVIII assay when calibrated with plasma standards include the following: Actin FS; Actin FSL; Auto APTT; Cephascreen, CK Prest; Pathromtin SL; PTT-A; SynthASil.
2. Chromogenic assays can be used when calibrated with plasma standards and locally verified.

N8-GP

N8-GP is an extended half-life recombinant B-domain-truncated FVIII with a site-specific 40 kDa polyethylene glycol moiety. Previous studies of plasma spiked to 20, 60 and 90 IU/dL of N8-GP have highlighted up to 30% overestimation above the target FVIII with some chromogenic assays and significant underestimation, by 50%, in one-stage assays with APTT-SP, a reagent with silica



activator.^{34,35} A recent study showed that silica-based activators can cause decelerated activation of the N8-GP by thrombin in comparison to native FVIII, and this may be the cause of underestimation of activity by one-stage clotting assay using silica-based reagents such as APTT-SP, STA-PTT Automate and TriniCLOT aPTT HS.³⁶

Recommendations for monitoring N8-GP:

1. APTT reagents which can be used in a one-stage clotting FVIII assay when calibrated with plasma standards and locally verified include the following: Actin, Actin FS, Pathromtin SL, SynthAFax and DG Synth.
2. One-stage assays unsuitable for monitoring N8-GP: APTT-SP, STA-PTT Automate and TriniCLOT aPTT HS (underestimates).
3. Chromogenic assays suitable for use include the following: Coatest, Coamatic, Siemens and Biophen.

Jivi

Jivi is a PEGylated B-domain-deleted recombinant human coagulation factor VIII, site-specific pegylated with a 60 kDa branched polyethylene glycol (two 30 kDa PEG) moiety. The molecular weight of the protein is approximately 234 kDa. The SPC states that factor VIII activity results by one-stage clotting assays can be significantly affected by the APTT reagent and the reference standard used and that there are significant discrepancies between assay results obtained by one-stage clotting assay and the chromogenic assay.³⁷ Based on results from a field study, the manufacturer indicated that some silica-based one-stage assays (e.g. APTT-SP, STA-PTT) may underestimate and some reagents, for example with kaolin-based activators, may overestimate activity. The manufacturer recommends laboratories intending to measure Jivi activity should check their procedures for accuracy.³⁷

A field study has been reported in which 52 laboratories in 10 countries assayed pooled haemophilic plasma samples spiked with Jivi at 4.3, 37.5 or 87.8 IU/dL.³⁸

Chromogenic assays in 16 laboratories recovered on average 104%–117% of target based on potency. One-stage assays were performed by 49 centres; Actin FS assays overestimated by >25% at all three levels, and CK Prest assays overestimated by >25% for samples containing four and 37 IU/dL but were close to target for the sample containing 87 IU/dL. One-stage assays with PTT-A (Stago) and APTT-SP (Werfen) significantly underestimated FVIII by an average of >75% of target across all three levels of Jivi. In a second part to the field study, the same centres performed one-stage assays with SynthASil ($n = 52$) and Pathromtin SL ($n = 51$) which were provided to participants and combined with a range of deficient plasmas, calibrators and analysers locally with acceptable results.

Recommendations for monitoring Jivi

1. APTT reagents which can be used in a one-stage clotting FVIII assay when calibrated with plasma standards include the following: Actin FSL, Pathromtin SL and SynthASil.

2. APTT reagents unsuitable for use in one-stage clotting FVIII assays include Actin FS, CK Prest (which overestimate) and APTT-SP, and PTT-A (which underestimate).
3. Chromogenic assays suitable when calibrated with plasma standards, traceable to the WHO International Standard for Plasma FVIII include the following: Coamatic, Electrachrom and Siemens.

Afstyla

This single-chain recombinant FVIII is a molecule in which most of the B-domain occurring in wild-type, full-length factor VIII and four amino acids of the adjacent acidic a3 domain is removed (amino acids 765–1652 of full-length factor VIII). The newly formed linkage of the heavy and light chain of factor VIII introduces a new N-glycosylation site. As the furin cleavage site presents in wild-type factor VIII between the B-domain and the a3 domain was removed, Afstyla is expressed as a single-chain factor VIII molecule.

The manufacturer recommends assay by chromogenic assay. The SPC³⁹ also indicates this but also states that the one-stage clotting assay result underestimates the factor VIII activity by approximately 45% compared with chromogenic assays and if the one-stage assay used, the result can be multiplied by a factor of 2. This strategy of using conversion factors to adjust assay results is not recommended by UKHCDO.⁴⁰

A field study using plasma spiked with 4, 30, 60 and 100 IU/dL Afstyla reported a consistent underestimation by 45% at all levels using one-stage clotting FVIII assays. Chromogenic assays in six laboratories recovered the expected value.⁴¹ A separate study comparing one-stage clotting FVIII assays using six APTT reagents (SynthASil, APTT-SP, SynthAFax, Actin FS, Actin FSL and Pathromtin SL) in plasma spiked with Afstyla reported greater variability between APTT reagents than in the field study, particularly at lower concentrations of FVIII.⁴²

Recommendations for monitoring Afstyla

1. One-stage clotting FVIII assays are not recommended for monitoring Afstyla. The use of conversion factors is considered unsuitable by the UKHCDO.
2. The Biophen FVIII Chromogenic assay can be used when calibrated with plasma standards traceable to the WHO International Standard for Plasma FVIII. Other chromogenic assays may be suitable but require local verification.

Adynovi

Adynovi is a pegylated full-length recombinant FVIII molecule protein, Advate, with 20 kDa polyethylene glycol, and the pegylation is not site-specific. Adynovi is marketed as Adynovate in some regions, and the potency is assigned by the one-stage assay. In contrast, in the EU/UK the potency label is by chromogenic assay.

The SPC for this product makes no specific recommendation of activity assay but stated that a field study has indicated that plasma



factor VIII levels can be monitored using either a chromogenic substrate assay or a one-stage clotting assay routinely used in clinical laboratories.⁴³

A field study has been published in which 35 laboratories in 12 countries returned one-stage clotting FVIII assay data, and 11 centres in eight countries returned chromogenic assay results.⁴⁴ Haemophilic plasma was spiked with Adynovate at 5, 20 or 80 IU/dL. Results of chromogenic assays and one-stage assays were on average 113% (range 101%-124% for the three samples) and 116% (range 92%-129%) of target based on stated potency, respectively. The following chromogenic kits were used: Siemens, Electrachrom and Coamatic. One-stage assays participants used a range of APTT reagents using different activators. There were no statistically significant differences between the results with different one-stage reagents. The authors concluded that either one-stage or chromogenic assay could be used, irrespective of the source of reagents. There was a second field study amongst eight Swiss laboratories which assessed use of one-stage and chromogenic assay and the possible use of a product specific calibrator.⁴⁵ In contrast to the study of Turecek, this article reported that FVIII assays in spiked samples containing 30-80 IU/dL overestimated relative to the labelled potency by 28%-32% for one-stage assay and 69%-82% for chromogenic. Overestimation was even higher at lower levels. All these differences were reduced if assays were calibrated with a product specific calibrator. The reasons for the different findings between these two studies are unknown. More data are required to support specific assay recommendations for this product.

Recommendation for monitoring Adynovi:

1. Until more conclusive data are available, we recommend that laboratories perform local verification using either one-stage or chromogenic assays.

5 | FACTOR IX PRODUCTS

In Europe, in accordance with the European Pharmacopoeia monograph for FIX,⁴⁶ potency labelling for all FIX products is by one-stage clotting assay, using APTT reagents. The pharmacopoeial monograph specifies that the measured potency should be within 80%-125% of the stated potency. Two commercial CE marked chromogenic assay kits are available for measurement of FIX activity, but neither chromogenic kit has been used for potency labelling of FIX products. Table 2 presents some FIX products licensed and available in the UK.

5.1 | Standard half-life FIX concentrates

5.1.1 | Plasma derived

Assay discrepancies relative to the International Standard (IS) for FIX, plasma or secondary or in-house plasma standards traceable to the plasma IS are rare. Results from NEQAS exercises illustrate good agreement.⁴⁷ In a two-centre study, the recovery of a plasma-derived FIX activity was found to be within acceptable limits for all APTT

reagents (SynthAFax, DG Synth, SynthASil, Actin, Actin FS, Actin FSL, Pathromtin SL and APTT-SP) and both the Rossix and Biophen FIX chromogenic assay kits tested.⁴⁸ All APTT reagents are therefore deemed to be suitable for monitoring replacement with plasma-derived FIX concentrates relative to a plasma reference standard.

Recommendation for monitoring plasma-derived FIX

1. One-stage FIX clotting assay verified for diagnostic use and calibrated with plasma standards traceable to the WHO International Standard for plasma FIX can be considered suitable for monitoring plasma-derived FIX concentrate infusions, unless there is specific evidence to the contrary.

5.1.2 | Full-length recombinant FIX

BeneFIX; Rixubis

BeneFIX and Rixubis are recombinant human coagulation FIX products. The summary of product characteristics for both products indicate FIX activity can be significantly affected by types of APTT reagent and the reference standard used in the one-stage clotting assay. However, no specific recommendation is provided.^{49,50}

Information on issues related to measurement of Rixubis is scant, with the US package insert stating FIX potency results can be affected by the type of APTT reagent used in the assay and that differences of up to 40% have been observed. In a comparative field study involving 30 laboratories, one-stage clotting activities of BeneFIX spiked plasma samples relative to in-house plasma reference standard were consistently overestimated, with kaolin-based reagent giving closer, but not significantly different, recovery than silica-based and ellagic acid reagents.⁵¹ In a two-centre study, DG Synth, Actin, Actin FS, Actin FSL and Pathromtin SL were found to give acceptable recovery to BeneFIX spiked plasma samples when SSC Lot 4 was used as the calibration standard.⁴⁸ In the same study, the Rossix chromogenic assay gave estimates close to target value, but Biophen chromogenic kit underestimated the activities of the spiked plasma activity sample.

Recommendation for monitoring full-length recombinant FIX

1. One-stage FIX clotting assay validated for diagnostic use and calibrated with plasma standards traceable to the WHO International Standard for plasma FIX can be considered suitable for monitoring recombinant FIX replacement therapy unless there is specific evidence to the contrary. Local verification of suitability of APTT reagent should also be carried out.

5.2 | Extended half-life recombinant FIX

Alprolix (rFIXFc)

Alprolix is an extended half-life recombinant factor IX molecule fused to the Fc portion of human immunoglobulin G1 (IgG1; rFIXFc). The summary of product characteristics states that 'When



using an in vitro thromboplastin time (aPTT)-based one-stage clotting assay for determining factor IX activity in patient's blood samples, plasma factor IX activity results can be significantly affected by both the type of APTT reagent and the reference standard used in the assay. This is of importance particularly when changing the laboratory and/or reagents used in the assay. Measurements with one-stage clotting assay utilizing a kaolin-based aPTT reagent will likely result in an underestimation of activity level.⁵² This recommendation was supported by results from a field study involving 30 laboratories, using 10 different APTT reagents and haemophilic plasma spiked with three different nominal concentrations (80, 20 or 5 IU/dL).⁵¹ A small laboratory study of 22 samples from five patients receiving Alprolix concluded that one-stage assays using Actin FSL, and the Rossix chromogenic FIX assay accurately recovered the expected FIX whereas up to 30% difference was observed by one-stage assays using Actin FS, Pathromtin SL, SynthASil, APTT-SP and SynthAFax and with Hyphen Chromogenic FIX assay.⁵³

Recommendations for monitoring Alprolix

1. APTT reagents which can be used in a one-stage clotting FIX assay when calibrated with plasma standards traceable to the WHO International Standard for plasma FIX include the following: Actin, Actin FS, Actin FSL, Pathromtin SL, Cephascreen and SynthASil.
2. APTT reagents unsuitable for use in one-stage clotting FIX assays include: CK Prest and PTT-A.
3. Chromogenic assay with FIX from Rossix.

Idelvion (rIX-FP)

Idelvion is a recombinant fusion protein linking FIX to albumin (rIX-FP). The summary of product characteristics states that 'when using an in vitro thromboplastin time (aPTT)-based one-stage clotting assay for determining Factor IX activity in patients' blood samples, plasma factor IX activity results can be significantly affected by both the type of aPTT reagent and the reference standard used in the assay. Measurement with a one-stage clotting assay using a kaolin-based aPTT reagent or Actin FS aPTT reagent will likely result in an underestimation of activity level. This is of importance particularly when changing the laboratory and/or reagents used in the assay'.⁵⁴

An international multicentre field study involved five centres participating in the phase I/II PROLONG-9FP clinical trial that tested Idelvion at concentrations of 25, 50 and 90 IU/dL, eight centres participating in the phase II/III clinical trial that tested Idelvion spiked at concentrations of 10, 25 and 62.5 IU/dL and 21 samples from patients in the phase II/III clinical trial. The study concluded that all samples demonstrated comparable results with a range of APTT reagents; however, Actin FS and CK Prest underestimated FIX by up to 50%.⁵⁵ A laboratory study of 18 samples

from three patients treated with Idelvion confirmed an underestimation of more than 30% with Actin FS but also observed a similar level of underestimation with Actin FSL and overestimation of more than 30% with SynthAFax and both Rossix and Hyphen chromogenic FIX assays.⁵³ Further studies are required to confirm these latter findings. The summary of product characteristics for Idelvion and a recent field study⁵⁶ indicated that plasma FIX activity results can be significantly affected by both the type of APTT reagent and the reference standard used in the assay and that a kaolin-based APTT reagent or Actin FS will be likely to give an underestimation of activity.

Recommendations for monitoring Idelvion

1. One-stage assay APTT reagents suitable for measuring Idelvion: Pathromtin SL, SynthASil.
2. APTT reagents unsuitable for measuring Idelvion: CK Prest, Actin FS (underestimates) SynthAFax (overestimates).
3. Chromogenic FIX assays unsuitable for measuring Idelvion: Rossix and Hyphen (both overestimate).

Refixia

Refixia is an extended half-life recombinant FIX with a 40 kDa polyethylene glycol moiety attached to the FIX activation peptide by site directed glycopegylation.⁵⁷ Inconsistencies between APTT reagents in the monitoring of Refixia have been reported^{47,48} due to enhanced activation of the PEGylated product.⁵⁸ The summary of product characteristics states 'Due to the interference of polyethylene glycol (PEG) in the one-stage clotting assay with various aPTT reagents, it is recommended to use a chromogenic assay (e.g. Rox Factor IX or Biophen) when monitoring is needed. If a chromogenic assay is not available, it is recommended to use a one-stage clotting assay with an aPTT reagent (e.g. Cephascreen) qualified for use with Refixia. For modified long-acting factor products, it is known that the one-stage clotting assay results are highly dependent on the aPTT reagent and reference standard used. For Refixia some reagents will cause underestimation (30%-50%), while most silica containing reagents will cause severe overestimation of the factor IX activity (more than 400%). Therefore, silica-based reagents should be avoided. Use of a reference laboratory is recommended when a chromogenic assay or a qualified one-stage clotting assay is not available locally'.⁵⁹ The EMA licensing assessment report indicated that using one-stage clotting assay, SynthAFax is the most appropriate APTT reagent for potency labelling and clinical monitoring and that there is an overestimation of activity using most silica-based reagents such as PTT automate (>500%) and Pathromtin SL (>2000%) and an underestimation using Actin FS.⁶⁰ A qualification study concluded that Refixia could be accurately measured by one-stage assay using Cephascreen and Chromogenic assays with Hyphen and Rossix.⁶¹

Recommendations for monitoring Refixia



1. APTT reagents suitable for measuring Refixia: DG Synth, SynthAFax, Cephascreen.
2. APTT reagents unsuitable for measuring Refixia: Actin FS, Actin, Actin FSL, SynthASil, Pathromtin SL, APTT-SP.
3. Chromogenic FIX assays suitable for monitoring Refixia: Hyphen, Rossix.

6 | CONCLUSION

When establishing assays for monitoring replacement therapy, especially for the extended half-life products, it is important to assess the suitability of the reagents and kits used. A review of the relevant field studies can be helpful, but it is essential to carry out local verification of the assay. Engagement with the clinicians is critical as increasingly different assays may need to be undertaken for the various products. Internal monitoring and participation in quality assurance exercises and regular internal monitoring will ensure the continuously good performance of assay.

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DISCLOSURE

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SUPPORTING INFORMATION

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

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