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ORIGINAL ARTICLE

Qualification of a select one-stage activated partial thromboplastin time-based clotting assay and two chromogenic assays for the post-administration monitoring of nonacog beta pegol

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Essentials

- Nonacog beta pegol (N9-GP) is an extended half-life, recombinant human factor IX (FIX).
- One-stage clotting (OSC) and chromogenic FIX activity assays were assessed for N9-GP recovery.
- OSC STA®-Cephascreen®, ROX FIX and BIOPHEN FIX chromogenic assays were qualified for N9-GP.
- Other extended half-life factor products should be assessed in a similar way prior to approval.

Summary. *Background:* Nonacog beta pegol (N9-GP) is an extended half-life, glycoPEGylated recombinant human factor IX that is under development for the prophylaxis and treatment of bleeding episodes in hemophilia B patients. Considerable reagent-dependent variability has been observed when one-stage clotting assays are used to measure the recovery of recombinant FIX products, including N9-GP. *Objective:* To qualify select one-stage clotting and chromogenic FIX activity assays for measuring N9-GP recovery. *Methods:* The accuracy and precision of the one-stage clotting assay (with the STA-Cephascreen activated partial thromboplastin [APTT] reagent) and the ROX Factor IX and BIOPHEN Factor IX chromogenic assays for measuring N9-GP recovery were assessed in N9-GP-spiked hemophilia B plasma samples in a systematic manner at three independent sites, with manufacturer-recommended protocols and/or site-specific assay setups, including different instruments. *Results:* For each of the three FIX activity assays qualified on five different reagent–instrument systems, acceptable intra-assay and interassay accuracy and precision, dilution integrity, reagent robustness and freeze–thaw and short-term sample stabilities were demonstrated. The STA-Cephascreen assay showed a limited reportable range at one of the three qualification sites, and the BIOPHEN Factor IX assay showed suspect low-end sensitivity at one of the three qualification sites. An individual laboratory would account for these limitations by adjusting the assay's reportable range; thus, these findings are not considered to impact the respective assay qualifications. *Conclusion:* The one-stage clotting assay with the STA-Cephascreen APTT reagent, the ROX Factor IX chromogenic assay and the BIOPHEN Factor IX chromogenic assay are considered to be qualified for the measurement of N9-GP in 3.2% (0.109 M) citrated human plasma.

Keywords: blood coagulation tests; hemophilia B; N9-GP; nonacog beta pegol; recombinant factor IX.

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Introduction

Hemophilia B (HB) is a blood disorder characterized by functional coagulation factor IX deficiency. Recombinant FIX (rFIX) products are administered as replacement therapy to afflicted individuals. Modification of rFIX products, e.g. by glycoPEGylation, can extend the half-life of

rFIX molecules [1]. These longer-acting coagulation factor products have the potential to improve the quality of life of individuals with HB by decreasing injection frequency and, potentially, improving treatment compliance.

Nonacog beta pegol (N9-GP) is a glycoPEGylated human rFIX molecule that is currently under development for the prophylaxis and treatment of bleeding episodes in HB patients. N9-GP has an extended single-dose half-life of 93 h [2], and has shown better recovery and higher FIX activity levels than other FIX molecules [1,3,4]. Furthermore, N9-GP has shown efficacy and tolerability in previously treated pediatric, adolescent and adult HB patients in phase III clinical trials [1,5–7].

FIX one-stage activated partial thromboplastin time (APTT)-based clotting assays (OSCs) and chromogenic activity assays can be used to monitor post-infusion FIX activity levels during FIX replacement therapy in HB patients. Of the two types of assay, OSCs are most commonly used for measurement of FIX activity in the clinic [8]. Variability in FIX activity results from OSCs has been observed when some of the newer recombinant FIX products, including RIXUBIS, ALPROLIX, and IDELVION, have been assessed [9–12]. The observed variability appears to be dependent on the types of APTT reagent and reference standard used, but other factors may also contribute. For RIXUBIS, FIX activity differences of up to 40% have been observed [10], and for IDELVION, differences of ~ 32–50% have been reported [12,13]. Recovery of ALPROLIX activity in OSCs was variable in a reagent-dependent manner, with non-parallelism leading to higher variability at lower concentrations and between testing laboratories [9]. PEGylated factor products have also been associated with reagent-dependent recovery variations in these assays [14]. These discrepancies in the prevalent OSCs are so significant that the Scientific and Standardization Committee (SSC) of the ISTH has recommended that manufacturers define methods that can recover their molecules accurately [15]. In addition, it is a major concern for regulatory bodies, who request a reliable and accurate assay for measuring drug recovery in treated patients.

An alternative to the OSC is the chromogenic FIX activity assay. Studies have suggested that the effect of PEGylation on FIX recovery when chromogenic assays are used is less variable than with OSCs [11,14,16]. However, these assays are currently not widely used in Europe or the USA, despite the use of chromogenic FVIII assays in these regions. Chromogenic FIX assays have only recently become commercially available. The assays are Conformité Européenne-marked in Europe, but are available in the USA for research use only. The US Medical and Scientific Advisory Council of the American Hemophilia Society recommends that laboratories performing factor assays routinely on hemophilic patients strongly consider the addition of FVIII and FIX chromogenic assays upon approval by the US Food and Drug Administration (FDA) [17].

In previous assay comparison studies using spiked samples, N9-GP FIX activity was evaluated with OSCs and chromogenic assays [14,16]. These studies showed that most APTT reagents were affected by PEGylation, causing either overestimation or underestimation; however, specific APTT reagents (e.g. SynthAFax, DG Synth, and STA-Cephascreen) and commercially available chromogenic assays showed minimal PEG interference [14,18–20].

The study presented here was designed to qualify select FIX assays that had been previously identified as accurately recovering N9-GP in citrated human plasma [14,19] in a systematic manner by investigating each reagent system for various parameters at three independent sites, with manufacturer-recommended protocols and/or site-specific assay setups, in order to assess whether the assays can produce reproducible and accurate results. Of the three APTT reagents previously shown to recover N9-GP adequately, only the STA-Cephascreen APTT reagent was included, as an FDA-approved instrument application for the STA-R Evolution is available. At the time when the study was performed, DG Synth was not FDA-approved and there was no application for the SynthAFax APTT reagent on the IL ACL-TOP family of instruments, although the reagent itself is FDA-approved [14,18,19]. The outcomes of this study will provide guidance for clinical laboratories to determine suitable coagulation assays and reagent–instrument systems for measuring FIX activity in samples containing N9-GP.

Materials and methods

Study design

The *in vitro* recovery of N9-GP in citrated human plasma was assessed at each of three independent sites with three different FIX activity assays. The assays were the STA-Cephascreen OSC with STA-Deficient IX plasma (Diagnostica Stago, Gennevilliers, France [Stago (F)]), the ROX Factor IX chromogenic assay (Rossix AB, Mölndal, Sweden [Rossix (S)]), and the BIOPHEN Factor IX chromogenic assay (Hyphen Biomed SAS, Neuville-sur-Oise, France [Hyphen (F)]). Participating sites were supplied with critical assay reagents of the same source and lot number. Similarly, calibrator and quality control (QC) material of the same lot number were provided for STA-Cephascreen and BIOPHEN Factor IX assays by Stago (F) and Hyphen(F), respectively. For the ROX Factor IX assay, manufacturer-specific calibration and QC materials were not available, and, instead, CRYOcheck reference plasma and controls were used (Data S1).

Sample preparation

Test samples were prepared by Esoterix (Englewood, CO, USA [Esoterix (E)]), and provided frozen to individual

qualification sites. Samples were prepared by spiking N9-GP (Novo Nordisk A/S, Bagsvaerd, Denmark) into 3.2% (0.109 M) citrated congenitally FIX-deficient plasma (George King Biomedical, Overland Park, KS, USA) at concentrations of 0.05 IU mL^{-1} (test sample-1 [TS-1]), 0.15 IU mL^{-1} (test sample-2 [TS-2]), 0.30 IU mL^{-1} (test sample-3 [TS-3]), 1.20 IU mL^{-1} (test sample-4 [TS-4]), and 2.50 IU mL^{-1} (diluted working stock solution [DIL-TS]), according to the provided potency label. To verify test sample concentrations, samples were tested in duplicate at three dilutions in a validated FIX OSC that was used for testing phase I–III N9-GP clinical samples. The samples (TS-1 to TS-4) were recovered at 0.049, 0.140, 0.313 and 1.200 IU mL^{-1}, respectively, confirming sample preparation and recovery of N9-GP FIX activity. Prepared test samples were frozen and stored at − 70 °C before being shipped on dry ice to participating qualification sites.

FIX OSC and chromogenic assays

Each of the FIX OSC and chromogenic assay reagents was tested at three independent sites. The site-specific assay setups were established in a manner consistent with respective laboratory practice standards (Table 1). Where available, manufacturer-provided or recommended instrument setups and/or protocols were used. Further method-specific details on each of the site-specific reagent–instrument systems are provided in Data S1.

Assessment of qualification parameters

With the exception of freeze–thaw and long-term sample stability, qualification parameters were assessed at each participating qualification site.

Table 1 Factor IX activity assays: one-stage activated partial thromboplastin time-based clotting assay (OSC) and chromogenic assay reagent systems

Reagent	Instrument	Qualification site
STA-Cephascreen	STA-R Evolution	Esoterix (E)
STA-Deficient IX	STA-R Evolution	Esoterix (P)
plasma Stago (F) (OSC)	STA-R Evolution	Stago (F)
ROX Factor	Manual assay setup	Esoterix (E)
IX Rossix (S)	Sysmex CS-5100i	RHH (U)
(chromogenic)	Manual assay setup	Rossix (S)
BIOPHEN Factor	STA-R Evolution	Esoterix (E)
IX Hyphen (F)	Sysmex CS-5100i	RHH (U)
(chromogenic)	Sysmex CS-5100i	Hyphen (F)

Esoterix (E), Esoterix (Englewood, CO, USA); Esoterix (P), Esoterix (Phoenix, AZ, USA); Hyphen (F), Hyphen BioMed SAS (Neuville-sur-Oise, France); Rossix (S), Rossix AB (Mölndal, Sweden); RHH (U), Royal Hallamshire Hospital (Sheffield, UK); Stago (F), Diagnostica Stago (Gennevilliers, France).

Accuracy Five replicates of each test sample were assessed on each of the three independent assay runs. Intra-assay and interassay accuracy, expressed as percentage relative error (%RE), was demonstrated either by comparing the mean FIX activity obtained for each of the qualification runs (intra-assay accuracy), or the mean FIX activity obtained across all three qualification runs (interassay accuracy), with the mathematically calculated target concentration for each test sample. The acceptance range for intra-assay and interassay accuracy (%RE) was defined as ± 30% to reflect the published assay variability of native factors in existing OSC and chromogenic assay factor activity reagents [21,22].

Precision Intra-assay variability (percentage coefficient of variation [%CV]) was calculated for each of the three individual qualification runs, and interassay total imprecision (%CV) was calculated by using the standard deviation and mean of all 15 FIX activity values obtained over the three qualification runs. The acceptable range for intra-assay and interassay precision was defined as ≤ 20%.

Dilution integrity Three independent dilutions of DIL-TS (prepared from three separate vials of N9-GP material) were tested in singlicate on a single run. The acceptance criterion was defined as recovery of N9-GP FIX activity within ± 30% of the mathematical target (2.5 IU mL^{-1}).

Short-term sample stability on cold-block, ice, or bench-top Stability testing was conducted according to local laboratory sample handling practices. TS-1 and TS-4 were assessed either immediately after thawing (0-h time point) or after thawing and storage on cold-block, ice or bench-top (2-h, 4-h and 6-h time points). Three vials of each test sample were tested in singlicate on a single run. Acceptable recovery of FIX activity at each time point was defined as ± 20% of the mean FIX activity obtained at the 0-h time point.

Short-term sample stability on instrument For automated assays, stability was assessed for sample storage on board the analyzer. TS-1 and TS-4 were assessed either immediately after thawing (0-h time point), or after storage on the instrument (2-h, 4-h and 6-h time points). Three vials of each test sample were tested in singlicate on a single run. Acceptable recovery of FIX activity at each time point was defined as ± 20% of the mean FIX activity obtained at the 0-h time point.

Freeze–thaw sample stability This parameter was assessed at Esoterix (E) only. Three vials of TS-1 and TS-4 were subjected to an additional one, two or three freeze–thaw cycles, and then tested on each assay system.

For a given assay system, all freeze–thaw stability samples were tested on a single run. Acceptable recovery was defined as ± 20% of the respective interassay mean determined during accuracy and precision testing.

Long-term sample storage stability at − 70 °C Fresh batches of TS-1 (0.05 IU mL^{-1}) and TS-4 (1.2 IU mL^{-1}) were prepared to test long-term stability with each assay system. Testing was performed at Esoterix (E). Three aliquots each of TS-1 and TS-4 were tested in singlicate at the immediate time point. Acceptable long-term stability at the 1-month and 3-month time points was defined as recovery within ± 20% of the mean FIX activity obtained at the immediate time point.

Reagent robustness The effect of variability between different lots of assay kits (for chromogenic assays) or critical reagents (APTT reagents for the OSC) on assay accuracy and precision was assessed on an additional run of five replicates each of TS-1, TS-2, TS-3, and TS-4, with a reagent lot other than that used during accuracy and precision testing. Acceptable reagent robustness was a mean %RE value within ± 15% of the respective interassay mean obtained during the accuracy and precision testing, and an intrarun precision (%CV) at ≤ 20% for each test sample. At Hyphen (F), three replicates of the SSC/ISTH Secondary Coagulation Standard Lot no. 4 (National Institute for Biological Standards and Control, Ridge, UK; SSC Lot no. 4) were also tested on each accuracy and precision run.

Carryover (automated coagulation analyzers only) For automated assays, replicates of TS-1 and TS-4 were tested in the following order: $TS\text{-}1_1$, $TS\text{-}1_2$, $TS\text{-}1_3$, $TS\text{-}4_1$, $TS\text{-}4_2$, $TS\text{-}1_4$, $TS\text{-}4_3$, $TS\text{-}4_4$, $TS\text{-}1_5$, $TS\text{-}1_6$, $TS\text{-}1_7$, $TS\text{-}1_8$, $TS\text{-}4_5$, $TS\text{-}4_6$, $TS\text{-}1_9$, $TS\text{-}4_7$, $TS\text{-}4_8$, $TS\text{-}1_{10}$, $TS\text{-}4_9$, $TS\text{-}4_{10}$, and $TS\text{-}1_{11}$. Carryover analysis was performed with EP EVALUATOR CARRYOVER module software (Data Innovations, South Burlington, VT, USA) in accordance with Clinical and Laboratory Standards Institute (CLSI) EP10-A3 [23]. Results were entered in order of testing, and the software assessed whether the assay met the acceptability limits for carryover according to CLSI EP10-A3.

Data analysis

Data analysis was performed in Microsoft Excel.

The %RE was calculated as

$$\frac{\text{mean FIX activity-target FIX activity}}{\text{target FIX activity}} \times 100$$

The %CV was calculated as

$$\frac{\text{SD}}{\text{mean FIX activity}} \times 100$$

Results

One-stage STA-Cephascreen FIX assay

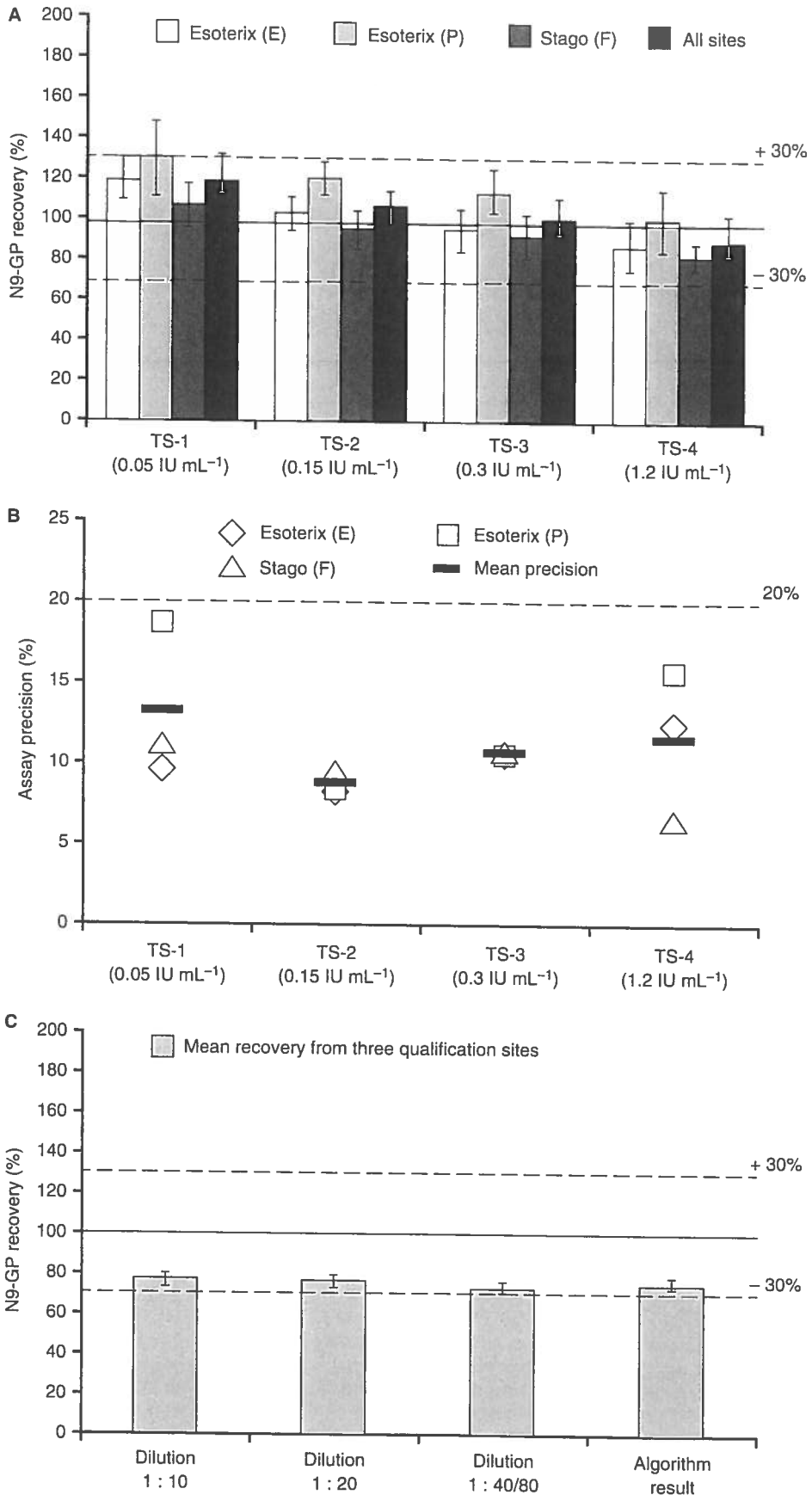
The STA-Cephascreen FIX OSC with STA-Deficient IX plasma was qualified for the measurement of N9-GP STA-Cephascreen FIX OSC qualification testing was conducted at all three sites with the manufacturer-recommended assay setup for the STA-R Evolution (Table 1).

Accuracy Mean intra-assay and interassay accuracy (% RE) fell within the established acceptance criteria (± 30% target value) for all test samples at each qualification site, with one exception (Fig. 1A). Across testing sites, the intra-assay accuracy ranged from − 24.6% (TS-4; Esoterix[E]) to 29.2% (TS-1; Esoterix [E]), and the interassay accuracy ranged from − 17.1% (TS-4; Stago [F]) to 30.0% (TS-1; [Esoterix (P)]), with the exception of one low-concentration test sample level, TS-1 at Esoterix (P), which showed a %RE of 42.8% in one of three accuracy and precision runs.

Precision Mean intra-assay precision and interassay precision (%CV) were acceptable (≤ 20%) for all test samples at each qualification site (Fig. 1B).

Dilution integrity Linearity across the three dilutions tested was demonstrated at all three sites (Fig. 1C). Dilution integrity was demonstrated at Esoterix (P), with a mean %RE of − 19.5% for 1 : 20, 1 : 40 and 1 : 80

Fig. 1. Evaluation of qualification parameters for use of the STA-Cephascreen one-stage clotting assay with STA-Deficient IX plasma for measuring FIX activity in nonacog beta pegol (N9-GP) samples. (A) Interassay accuracy was determined as percentage relative error of mean FIX activity obtained across three qualification runs at each site. The calculated means across all sites for each test sample level are also shown. The acceptable range was defined as ± 30% of the mathematical target value. Error bars represent standard deviation of the mean. (B) Interassay precision (percentage coefficient of variation) was determined by use of the standard deviation and mean of all FIX activity values obtained across three qualification runs. The acceptable range was defined as ≤ 20%. (C) Dilution integrity was assessed by testing three independent dilutions of diluted working stock solution, prepared from three separate vials of N9-GP material in singlicate on a single run. The acceptable target was defined as ± 30% of the mathematical target. Data represent the mean ± standard deviation for three sites. Qualification sites included Esoterix (Englewood, CO, USA [Esoterix (E)]), Esoterix (Phoenix, AZ, USA [Esoterix (P)]), and Diagnostica Stago (Gennevilliers, France; [Stago (F)]). TS-1, test sample-1; TS-2, test sample-2; TS-3, test sample-3; TS-4, test sample-4.



dilutions of N9-GP, and at Stago (F), with a mean %RE of – 21.8% for 1 : 10, 1 : 20 and 1 : 80 dilutions of N9-GP. Dilution integrity for N9-GP at Esoterix (E) failed to meet the acceptance criteria during original and repeat testing, demonstrating under-recovery of – 33.8% and – 32.4%, respectively.

Carryover analysis Carryover for the STA-Cephascreen assay with STA-Deficient IX plasma on the STA-R Evolution met the acceptance criteria as outlined in CLSI EP10-A3 [23] at all three qualification sites. Carryover results limits were – 0.002 IU mL^{-1} to 0.005 IU mL^{-1} (error limits were 0.008–0.015 IU mL^{-1}).

Reagent robustness Accuracy and precision for reagent robustness were demonstrated at both Esoterix (P) and Stago (F), with the %RE ranging from – 9.4 to 11.5%, and the %CV ranging from 1.5% to 11.3%. At Esoterix(E), the accuracy of a single test sample, TS-3, fell just outside of the ± 15% acceptance range, with a %RE of 15.4%.

Short-term, long-term and freeze–thaw stability results obtained with the one-stage STA-Cephascreen FIX assay are shown in Data S1.

Chromogenic ROX FIX assay

The ROX Factor IX chromogenic assay was qualified for the measurement of N9-GP The ROX Factor IX (Rossix[S]) chromogenic assay was automated on the Sysmex CS-5100i (Sysmex, Kobe, Japan) at Royal Hallamshire Hospital (Sheffield, UK [RHH (U)]), whereas the assay was performed manually with the 96-well plate format at Esoterix (E) and Rossix (S) (Table 1).

Accuracy Mean intra-assay and interassay accuracy (% RE) fell within the established acceptance criteria (± 30% target value) for all test samples at each qualification site (Fig. 2A). Intra-assay accuracy ranged from – 14.1% (TS-2; RHH[U]) to 29.9% (TS-3; RHH [U]), and interassay accuracy ranged from – 1.9% (TS-2; RHH [U]) to 17.3% (TS-3; Esoterix[E]).

Precision Mean intra-assay precision and interassay precision (%CV) were acceptable (≤ 20%) for all test samples at each qualification site (Fig. 2B).

Dilution integrity Dilution integrity for N9-GP at 2.5 IU mL^{-1} (DIL-TS) was demonstrated for all assay setups, with mean %RE values within the acceptance criteria of ± 30% (Fig. 2C). Mean %RE values were 8.6%, – 22.4% and – 6.6%, at Esoterix (E), RHH (U), and Rossix (S), respectively.

Carryover analysis Carryover analysis for ROX Factor IX automated on one Sysmex CS-5100i met the acceptance criteria as outlined in CLSI EP10-A3 [23]. The carryover result value was – 0.005 IU mL^{-1} (the error limit was 0.005 IU mL^{-1}).

Reagent robustness Accuracy (%RE) and precision (% CV) for a different lot of chromogenic reagents were demonstrated at RHH (U) and Rossix (S), with the % RE ranging from – 3.4% to 11.2%, and the %CV ranging from 1.6% to 7.7%. At Esoterix (E), TS-3 and TS-4 fell outside the ± 15% acceptance range, at – 15.5% and – 16.1%, respectively. The precision results for all samples at each qualification site fell between 1% and 8%.

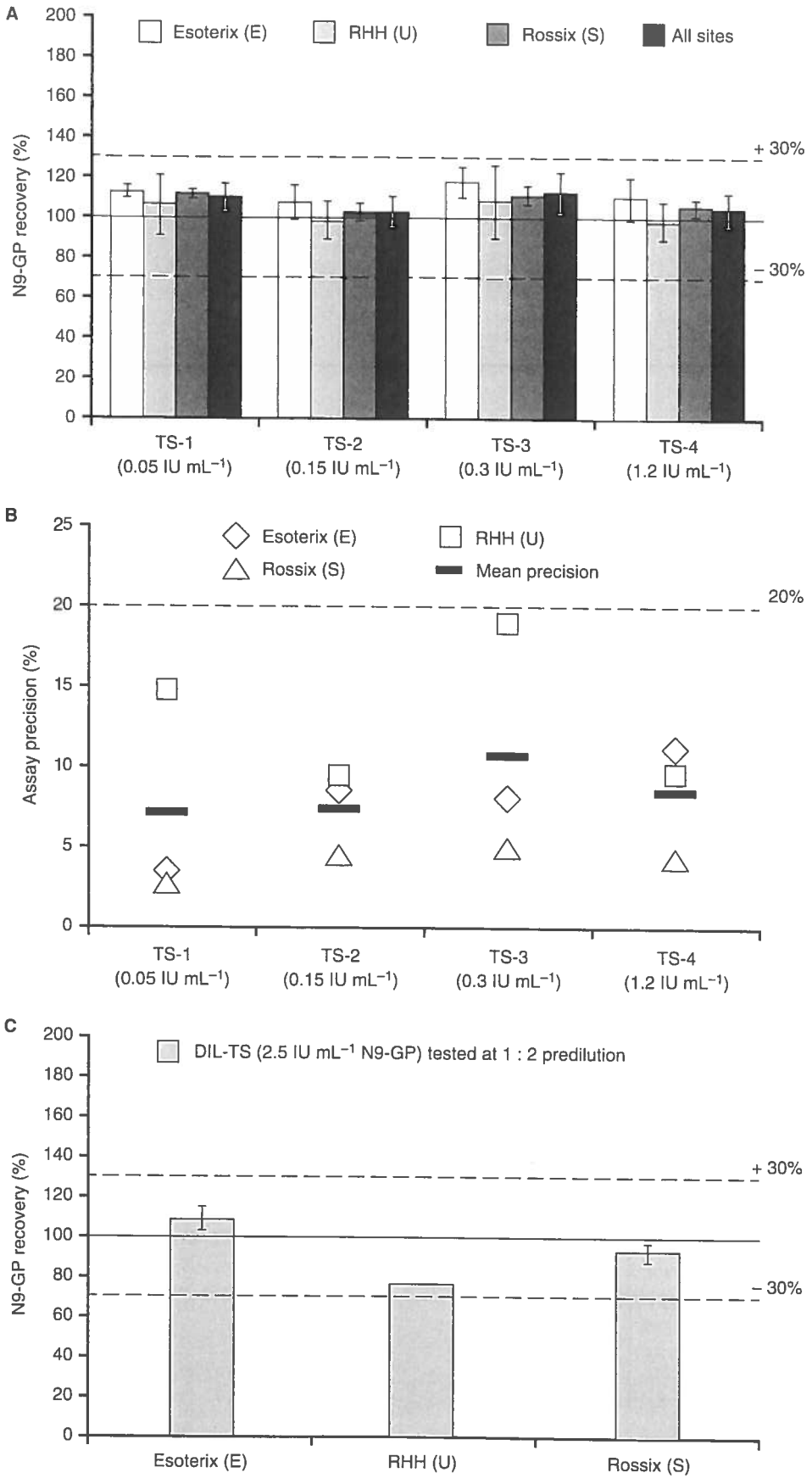
Short-term, long-term and freeze–thaw stability results obtained with the chromogenic ROX FIX assay are shown in Data S1.

Chromogenic BIOPHEN FIX assay

The BIOPHEN Factor IX chromogenic assay was qualified for the measurement of N9-GP The BIOPHEN Factor IX chromogenic assay was automated on the Sysmex CS-5100i at RHH (U) and Hyphen (F), and on the STA-R Evolution at Esoterix (E) (Table 1).

Accuracy Intra-assay and interassay accuracy (%RE) for N9-GP was acceptable (± 30% target value) across all qualification sites, with one exception (Fig. 3A). For intra-assay accuracy, acceptable %RE values ranged from – 26.8% (TS-3; RHH[U]) to 0.1% (TS-4; Esoterix [E]), and interassay accuracy ranged from – 24.6% (TS-3; Hyphen [F]) to – 3.9% (TS-4; Esoterix[E]). For TS-1 at RHH (U), mean intra-assay %RE values from two runs (– 36.4% and – 34.4%) and, as a result, the mean interassay %RE (– 31.8%) fell outside the acceptance criteria. Interestingly, this sample (TS-1) met the

Figure 2. Evaluation of qualification parameters for the use of the ROX Factor IX chromogenic assay for measuring FIX activity in nonacog beta pegol (N9-GP) samples. (A) Interassay accuracy was determined as percentage relative error of mean FIX activity obtained across three qualification runs at each site. The calculated means across all sites for each test sample level are also shown. The acceptable range was defined as ± 30% of the mathematical target value. Error bars represent standard deviation of the mean. (B) Interassay precision (percentage coefficient of variation) was determined by use of the standard deviation and mean of all FIX activity values obtained across three qualification runs. The acceptable range was defined as ≤ 20%. (C) Dilution integrity was assessed by testing three independent dilutions of diluted working stock solution (DIL-TS), prepared from three separate vials of N9-GP material in singlicate on a single run. The acceptable target was defined as ± 30% of the mathematical target. Data represent the mean ± standard deviation for three sites. Qualification sites included Esoterix (Englewood, CO, USA [Esoterix (E)]), Royal Hallamshire Hospital (Sheffield, UK; [RHH (U)]), and Rossix AB (Mölndal, Sweden; [Rossix (S)]). TS-1, test sample-1; TS-2, test sample-2; TS-3, test sample-3; TS-4, test sample-4.



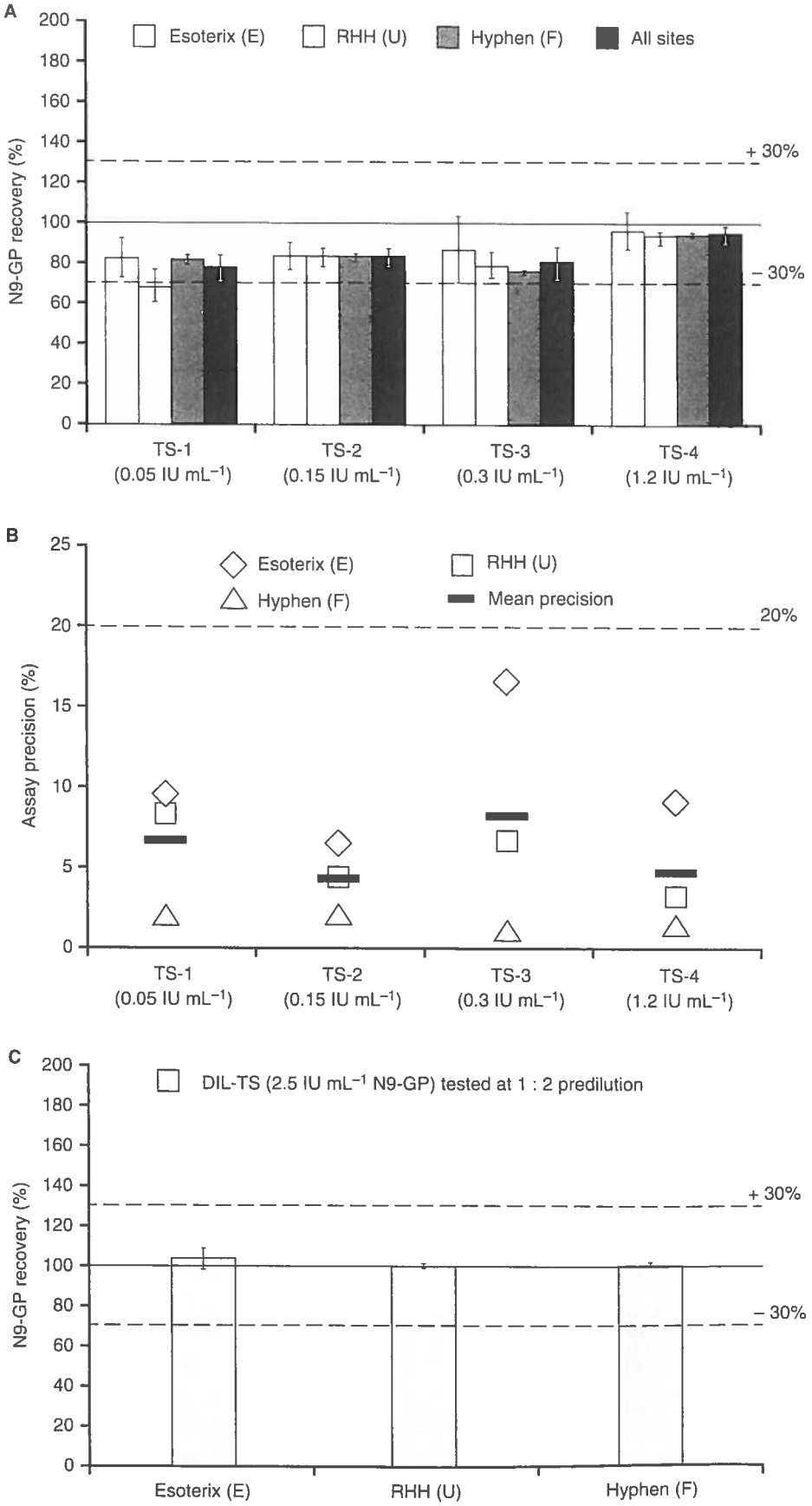


Fig. 3. Evaluation of qualification parameters for the use of the BIOPHEN Factor IX chromogenic assay for measuring FIX activity in nonacog beta pegol (N9-GP) samples. (A) Interassay accuracy was determined as percentage relative error of mean FIX activity obtained across three qualification runs at each site. The observed mean across all sites for each test sample level is also shown. The acceptable range was defined as ± 30% of the mathematical target value. Error bars represent the standard deviation of the mean. (B) Interassay precision (percentage coefficient of variation) was determined by use of the standard deviation and mean of all FIX activity values obtained across three qualification runs. The acceptable range was defined as ≤ 20%. (C) Dilution integrity was assessed by testing three independent dilutions of diluted working stock solution (DIL-TS), prepared from three separate vials of N9-GP material in singlicate on a single run. The acceptable target was defined as ± 30% of the mathematical target. Data represent the mean ± standard deviation. Qualification sites included Esoterix (Englewood, CO, USA [Esoterix (E)]), Royal Hallamshire Hospital (Sheffield, UK; [RHH (U)]), and Hyphen Biomed SAS (Neuville-sur-Oise, France; [Hyphen (F)]). TS-1, test sample-1; TS-2, test sample-2; TS-3, test sample-3; TS-4, test sample-4.

acceptance criteria on the same instrument–reagent system at Hyphen (F). Although within the acceptable range, recovery of N9-GP in the BIOPHEN Factor IX chromogenic assay fell consistently at the lower end of the ± 30% range. The intra-assay and interassay accuracy for the three replicates of SSC Lot no. 4 tested at Hyphen (F) were acceptable (%RE within ± 30% target value), with %RE values ranging from 10.3% to 12.7%.

Precision The mean intra-assay and interassay precision (%CV) for all test samples at each of the three qualification sites fell within the ≤ 20% range (Fig. 3B). The intra-assay precision and interassay precision (%CV) for SSC Lot no. 4 were also acceptable (≤ 20%), with the % CV ranging from 0.3% to 1.2%.

Dilution integrity Dilution integrity for N9-GP was demonstrated at all testing sites with mean %RE values of 3.4%, 0.1% and 0.8% for assay setups at Esoterix (E), RHH (U) and Hyphen (F), respectively (Fig. 3C).

Carryover analysis Carryover analysis for the BIOPHEN Factor IX assay on the Sysmex CS-5100i and the STA-R Evolution met the acceptance criteria as outlined in CLSI EP10-A3 [23]. Carryover result limits were 0–0.006 IU mL^{-1} (error limits were 0.002–0.013 IU mL^{-1}).

Reagent robustness Accuracy and precision for reagent robustness were demonstrated at RHH (U) and Hyphen (F), with the %RE ranging from − 5.9% to 13.8%, and the %CV ranging from 0.6% to 2.1%. At Esoterix (E), accuracy for the TS-2 and TS-3 samples fell outside the ± 15% range, at − 17.9% and 21.7%, respectively. Precision at this site was acceptable, ranging from 5.5% to 19.8%. At Hyphen (F), three replicates of SSC Lot no. 4 also showed reagent robustness, with %RE and %CV of 3.3% and 0.2%, respectively.

Short-term, long-term and freeze–thaw stability results obtained with the chromogenic BIOPHEN Factor IX assay are shown in Data S1.

A comparison of the interassay accuracy and precision results for each of the three assays assessed in this study is shown in Table 2.

Table 2 Summary of nonacog beta pegol qualification results obtained with the STA-Cephascreen one-stage clotting assay, the ROX Factor IX chromogenic assay and the BIOPHEN Factor IX chromogenic assays across three qualification sites

	STA-Cephascreen	ROX Factor IX	BIOPHEN Factor IX
Accuracy (interassay)	Mean % recovery	Mean % recovery	Mean % recovery
0.05 IU mL^{-1} (TS-1)	118.7	109.8	77.2
0.15 IU mL^{-1} (TS-2)	106.3	102.6	82.6
0.30 IU mL^{-1} (TS-3)	100.8	112.0	80.0
1.20 IU mL^{-1} (TS-4)	90.6	104.0	94.1
2.5 IU mL^{-1} (DIL-TS)	75.0	93.2	101.4
Precision (interassay)	%CV	%CV	%CV
0.05 IU mL^{-1} (TS-1)	16.2	8.9	10.9
0.15 IU mL^{-1} (TS-2)	13.0	8.4	4.5
0.30 IU mL^{-1} (TS-3)	14.1	12.3	12.2
1.20 IU mL^{-1} (TS-4)	15.0	9.8	5.8
2.5 IU mL^{-1} (DIL-TS)	9.2	15.0	3.1
Short-term stability			
On cold block	Acceptable*	Acceptable*	Acceptable*
On benchtop	Acceptable*	NA	Acceptable*
On instrument	Acceptable*	NA	Acceptable*
Freeze–thaw stability	Acceptable*	Acceptable*	Acceptable*

%CV, percentage coefficient of variation; DIL-TS, diluted working stock solution; NA, non-applicable; TS, test sample. The STA-Cephascreen one-stage clotting assay was performed with STA-Deficient IX plasma. *Results fell within ± 20% of the expected target.

Discussion

Accurate measurement of FIX activity for new, modified FIX products can be of concern, owing to the heterogeneity of the many commercially available APTT reagents and the significant discrepancies observed for some products [9–12,14,24]. To address this, it is essential to identify methods that allow accurate monitoring of the FIX activity of various FIX replacement products, and to provide this information as guidance to clinical laboratories. Furthermore, it is crucial for a manufacturer's assay used for product potency assignment to be in alignment with the clinical laboratory assay used for post-administration monitoring, to prevent underdosing or overdosing [24].

As a first of its kind, this study aimed to address the assay variability of a modified recombinant FIX product

by assessing and qualifying select reagent–instrument systems at multiple sites using various assay protocols. Specifically, this study focused on coagulation assays used for measuring FIX activity of N9-GP-containing samples. One FIX OSC and two chromogenic assays were evaluated for the measurement of N9-GP across multiple institutions and instrument systems (Table 2). The reagents investigated were chosen because they have previously shown acceptable N9-GP recovery in citrated human plasma. [14,19] In this study, we focused on the STA-Cephascreen APTT reagent, for which an FDA-approved instrument application for a commonly used coagulation analyzer, the STA-R Evolution, was available [14,18,19].

Several factors may contribute to the observed variability of current coagulation assays: different combinations of reagents and instruments, differences in reference or standard plasmas [25], assay buffer versus diluent (factor-deficient plasma versus sodium chloride), single or dual (high and low) calibration curves, and whether or not data are extrapolated by use of a calibration curve. In addition, recently published studies have shown that glycoPEGylation affects APTT assays by interfering with contact activators (e.g. silica); however, other etiologies are also likely [26,27]. The chromogenic assays are considered to be more robust, but even these assays are prone to variability.

All reagent and instrument systems in this study were systematically tested and qualified for the measurement of FIX activity in samples containing N9-GP. Although some limitations were identified (described below), these were determined to be manageable. For the STA-Cephascreen OSC, initial and repeat dilution integrity testing for DIL-TS (2.5 IU mL^{-1} N9-GP) failed to meet the acceptance criteria of %RE within ± 30% at one of three qualification sites. However, linearity across the three dilutions was demonstrated. As the reportable range of this assay for N9-GP would be truncated to ≤ 1.2 IU mL^{-1} (TS-4) at this site, and as most clinical samples are expected to have FIX activity lower than this level, this observation does not impact on the overall qualification. For the BIOPHEN Factor IX chromogenic assay, the recovery of N9-GP at all qualification sites was consistently at the lower end of the ± 30% acceptance range. In addition, at one of the three qualification sites, the intra-assay and interassay accuracy for TS-1 (0.05 IU mL^{-1}) fell outside the acceptance range in two of three runs. The low-end sensitivity for this qualification site with the Sysmex CS-5100i is therefore unacceptable, despite the acceptable performance of the same instrument–reagent system at another qualification site. As the observed degree of under-recovery of N9-GP at trough levels is not clinically significant, this failure does not impact on the qualification method. Although undesirable, both of the above-mentioned limitations could be addressed at the local laboratory level by adjusting the reportable range of the assay, thus limiting the impact on the respective assay qualification.

Normally, it is standard laboratory practice to perform reagent lot comparisons before implementing a new reagent lot for clinical use. Despite the lot-specific failures of reagent robustness at one of three sites for each assay, these would not impact the qualification, because all limitations described here can be addressed at the local laboratory level. Furthermore, it is recommended that testing for these limitations is incorporated as routine practice at individual laboratories.

Another possible limitation of the study is the use of N9-GP-spiked plasma samples instead of samples from patients treated with N9-GP for the qualification analyses. However, as demonstrated in a recent study in which *in vitro* FIX-deficient plasma samples spiked with N9-GP and *in vivo* post-administration samples from N9-GP-treated patients behaved similarly in OSCs and chromogenic FIX activity assays, similar results would be expected for patient samples [18].

Overall, the results from this study support the qualification of the following reagent–instrument systems for measurement of N9-GP FIX activity in 3.2% citrated human plasma: FIX OSC with STA-Cephascreen and with STA-Deficient IX plasma on the STA-R Evolution; FIX chromogenic assays with ROX Factor IX on the Sysmex CS-5100i (automated) or manual setup; and the BIOPHEN Factor IX chromogenic assay on the STA-R Evolution and Sysmex CS-5100i. It should be noted that appropriate validation by individual clinical laboratories according to local regulatory requirements is necessary prior to implementation of any of these qualified assays to measure N9-GP activity. In future, this investigation could be expanded to assess and qualify additional reagent–instrument systems for the measurement of N9-GP. It is recommended that other novel extended half-life factor products undergo assessments similar to those presented here.

Addendum

S. Tiefenbacher and M. Ezban designed the study. R. Bohra, A. Bowyer, J. Amiral, S. Kitchen, A. Lochu, and S. Rosén performed the research. S. Tiefenbacher and R. Bohra analyzed the data. All authors contributed to the writing and review of the manuscript, and approved the final version.

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Disclosure of Conflict of Interests

S. Tiefenbacher is employed by Colorado Coagulation, Laboratory Corporation of America Holdings, Englewood, CO, USA, and has received consultancy and speaker fees from Novo Nordisk. R. Bohra, at the time of data acquisition, was an employee of Colorado Coagulation, and was later employed at Novo Nordisk A/S, Måløv, Denmark. J. Amiral serves as Scientific Consultant for Hyphen BioMed SAS, Neuville-sur-Oise, France. A. Bowyer has received funding from Novo Nordisk to support meeting presentations. S. Kitchen has received consultancy and speaker fees from Novo Nordisk. A. Lochu is employed by Diagnostica Stago, Inc., Gennevilliers, France. S. Rosén is a Senior Consultant to Rossix AB, Mölndal, Sweden, and has received speaker fees from Novo Nordisk. M. Ezban is employed by Novo Nordisk A/S, Bagsvaerd, Denmark.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Supplementary methods.

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