Features of a new chromogenic kit for determination of FIX activity in plasma and FIX concentrates.

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Background

FIX activity in plasma and concentrates is currently determined with clotting methods. The accuracy may be compromised due to low-resolution dose-response curves. New rFIX variants may be a challenge regarding suitability of clotting methods.

Objective

Development of a chromogenic kit, Rox Factor IX, for determination of FIX activity from 2 IU/mL down to 0.01 IU/mL in plasma and for potency assignment of FIX concentrates.

Method principle

FIX is activated by FXIa with simultaneous activation of FX in the presence of FVIII, phospholipid and Ca^{2+} followed by FXa hydrolysis of Z-D-Arg-Gly-Arg-pNA. Similar to clotting methods, FVIII is activated by thrombin generated in the assay. A heparin antagonist and a fibrin polymerization inhibitor are included in the reagents. There is no use of FIX deficient plasma.

Results

Two plasma samples with 0.01 IU/m were accurately determined and showed a typical response of 30 mA405/min above the blank in a microplate assay.
Overlapping dose-response curves were obtained for plasma diluted in kit buffer $\pm$ FIX deficient plasma. Analyses of low (L) and normal (N) control plasma showed intra and inter assay CV of 4 % and 9 % (L) and 5 % and 5 % (N), respectively, in a manually performed method.
Analysis of artificially prepared plasma samples with 0.01 – 1.8 IU/mL FIX activity (assayed at 1:20 or 1:80 dilutions) showed a quantitative recovery with R^2 = 0.998 and slope 1.02.
Spiking of FIXa into the 4th IS showed no interference at $\leq$ 1%.
Analysis of pdFIX concentrates vs the 4th IS (07/182) showed a recovery in agreement with assigned values and the chromogenic kit method correlated well with a FIX clotting method (ACL 9000) with R^2 = 0.9 (n = 53). Altogether, the performance of Rox Factor IX should make it an interesting alternative to FIX clotting methods.

POSTER INCLUDED

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Features of a new chromogenic kit for determination of FIX activity in plasma and FIX concentrates.

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Background

Factor IX (FIX) activity in plasma and concentrates is currently determined with clotting methods. The accuracy of clotting methods may be compromised due to low-resolution dose-response curves. Furthermore, new recombinant FIX variants (rFIX) may constitute a challenge regarding suitability of clotting methods.

Objective

To evaluate a new chromogenic kit (Rox Factor IX) for determination of FIX activity from 2 IU/mL down to 0.01 IU/mL in plasma and for potency assignment of FIX concentrates.

Materials and Methods

Method Principle

FIX is activated by FXIa with simultaneous activation of FX in the presence of FVIII, phospholipids and Ca^{2+} followed by FXa hydrolysis of Z-D-Arg-Gly-Arg-pNA. Similar to clotting methods, FVIII is activated by thrombin generated in the assay. A heparin antagonist and a fibrin polymerization inhibitor are included in the reagents. There is no use of FIX deficient plasma.

Chromogenic Method

The method was performed manually (Table 1). Absorbance readings were made using a T-Max microplate reader (MolecularDevices). Evaluations were made with Log-log and 4-parametric curve-fitting models.

Correlation Study

Artificially prepared plasma samples (n= 30) and plasma samples from normal healthy donors (n=36) were analysed.
Artificial plasma samples were prepared by diluting normal reference plasma (Precision Biologics) with FIX deficient plasma (Precision Biologics) and by spiking of the 4th IS FIX Concentrate, 07/182 (NIBSC) to the reference plasma. FIX potencies were assigned with Rox Factor IX and a one-stage clotting method. The one-stage clotting method was performed on ACL 9000 (Instrumentation Laboratory) using APTT Reagents (MediRox) and FIX deficient plasma.

Precision, Discrimination and Detection Limit

Samples were prepared by spiking of the 4th IS FIX Concentrate into FIX deficient plasma and analysed in five independent assay series (N=5) with four replicates in each series (n=20).
Four of the samples were potency assigned against a plasma standard diluted in diluent ± 5% FIX deficiency plasma, providing the same plasma concentration as a plasma sample diluted 1:20.

Effect of Factor IXa

To evaluate the effect of preactivation of FIX, different levels of the 1st IS FIXa, 97/562 (NIBSC), was added to the 4th IS FIX Concentrate.

Table 1: Rox Factor IX, Manual Method

Sample Dilution* (18-25°C)	25 µL
Reagent A (18-25°C)	25 µL
Preheating, 3-5 min at 37°C	
Reagent B (37°C)	150 µL
Activation, 8 min at 37°C	
FXa Substrate (37°C)	50 µL
Hydrolysis, 2 min at 37°C	
Citric Acid, 2% (18-25°C)	50 µL
Reagent A: human FVIII, human FX and a fibrin polymerization inhibitor.	
Reagent B: bovine FXIa, calcium chloride and phospholipids	

*Plasma samples with expected FIX activity ≥ 0.25 IU/mL were diluted 1:80
Plasma samples with expected FIX activity ≤ 0.25 IU/mL were diluted 1:20
FIX Concentrates were diluted to arrive within the standard range (0.5 – 25 mIU/mL)

Results

For conversion of IU/mL to % activity, multiply by 100.

Figure 1: FIX dose response for a plasma standard in the range 0.5 - 25 mIU/mL (plasma diluted 1:2000 – 1:40) presented in a Log-Log graph (n=4).

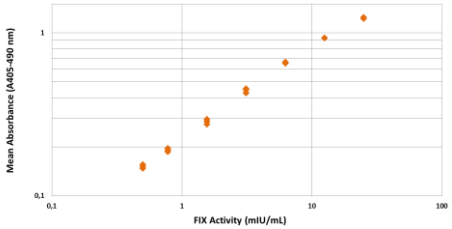


Figure 2: Parallel line evaluation of a pdFIX concentrate sample vs. the 4th IS FIX Concentrate in the range 0.5 – 4 mIU/mL, using 4 min hydrolysis at 37°C (n=3).

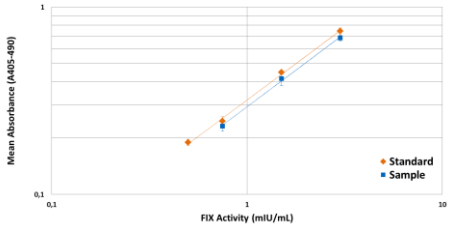


Figure 3: Correlation to a One-Stage Clotting method (n=66).

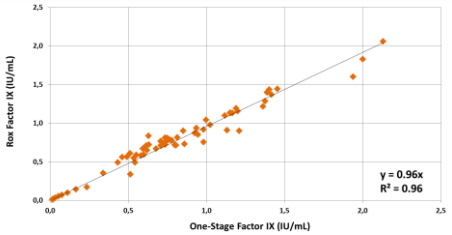


Figure 4: Assigned FIX potencies of artificially prepared plasma samples (n=30)

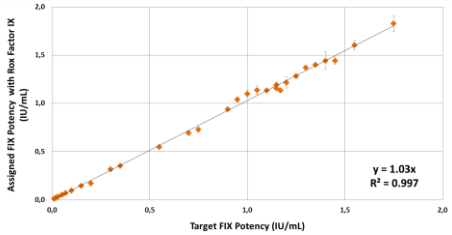


Table 2: Discrimination at relevant FIX deficiency classification levels (N=5, n=20).

Sample	Mean ± SD	± 2 SD
10 mIU/mL	10 ± 1	8 - 12
15 mIU/mL	15 ± 1	13 - 17
43 mIU/mL	43 ± 2	39 - 47
53 mIU/mL	53 ± 1	51 - 55
0.46 IU/mL	0.46 ± 0.01	0.44 - 0.48
0.54 IU/mL	0.54 ± 0.02	0.50 - 0.58

Table 3: Assigned values of plasma samples calculated against a standard diluted in diluent ± FIX deficient plasma (n=4).

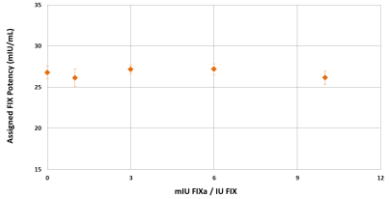
Sample	Diluent	Diluent + FIX deficient plasma
10 mIU/mL	9.6 ± 0.1	9.2 ± 0.1
20 mIU/mL	20 ± 0.4	20 ± 0.4
53 mIU/mL	54 ± 0.9	53 ± 0.9
0.46 IU/mL	0.46 ± 0.01	0.46 ± 0.01

Table 4: Within (n=20) and Between (N=5) Series CV%

Sample	Within Series	Between Series
10 mIU/mL	≤ 5%	≤ 9%
0.9 IU/mL	≤ 5%	≤ 5%

Detection Limit, arrived at from the apparent A405-490 obtained for the sample blank plus 3 standard deviations, is about 5 mIU/mL (0.5%) for plasma samples diluted 1:20 and 20 mIU/mL (2%) for plasma samples diluted 1:80 (N=5, n=20).

Figure 5: Assigned potencies of a FIX concentrate sample spiked with 0 - 10 mIU FIXa/IU FIX (n=3).



Conclusions

- Rox Factor IX provides a robust method suitable for analysis of Factor IX activity in human plasma and in FIX concentrates. The method makes no use of FIX deficient plasma.
- The kit method shows a high agreement and correlation with a one-stage clotting method (y=0.96, R^2=0.96, n=66).
- A proper discrimination is obtained at relevant FIX deficiency classification levels.
- Detection Limit is about 5 mIU/mL for plasma diluted 1:20.
- FIX concentrates diluted to 0.5 - 4 mIU/mL are suitably potency assigned using the bio assay parallel line evaluation.
- There is no interference of FIXa up to 10 mIU FIXa/IU FIX.

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