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

**Pia Bryngelhed, Per Rosén, Steffen Rosén**
**Rossix AB, Taljegardsgatan 3B, Mölndal, Sweden**

## 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 4$^{th}$ IS showed no interference at $\leq$ 1%.
Analysis of pdFIX concentrates vs the 4$^{th}$ 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**

Presented at the International Society on Thrombosis and Haemostasis (ISTH) XXIII Congress, Kyoto, Japan; July 23 - 28, 2011

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

Bryngelhed Pia, Rosén Per, Rosén Steffen
Rossix AB, Mölndal, Sweden

## 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).**

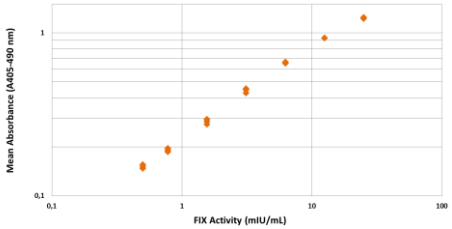



**Figure 2: Parallel line evaluation of a pdFIX concentrate sample vs. the 4th IS FIX Concentrate in the range 0.5 – 4 mIU/mL, using 4 min hydrolysis at 37°C (n=3).**

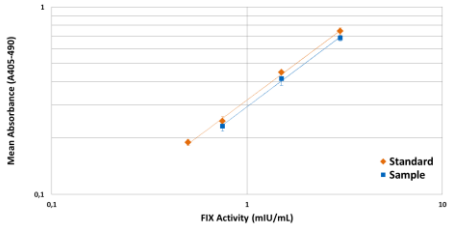



**Figure 3: Correlation to a One-Stage Clotting method (n=66).**

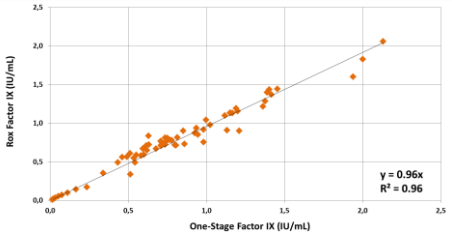



**Figure 4: Assigned FIX potencies of artificially prepared plasma samples (n=30)**

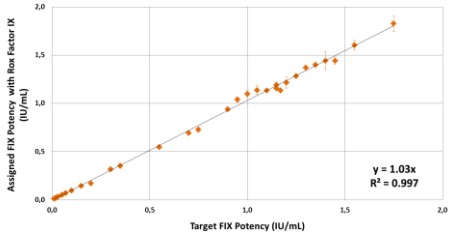



**Table 2: Discrimination at relevant FIX deficiency classification levels (N=5, n=20).**

| 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).**

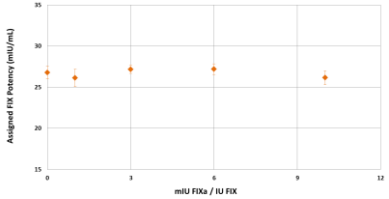
| Sample | Diluent | Diluent + FIX deficient plasma |
|---|---|---|
| **10 mIU/mL** | 9.6 ± 0.1 | 9.2 ± 0.1 |
| **20 mIU/mL** | 20 ± 0.4 | 20 ± 0.4 |
| **53 mIU/mL** | 54 ± 0.9 | 53 ± 0.9 |
| **0.46 IU/mL** | 0.46 ± 0.01 | 0.46 ± 0.01 |

**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.

Rossix