How to measure low levels of FVIII and IX

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Disclosures

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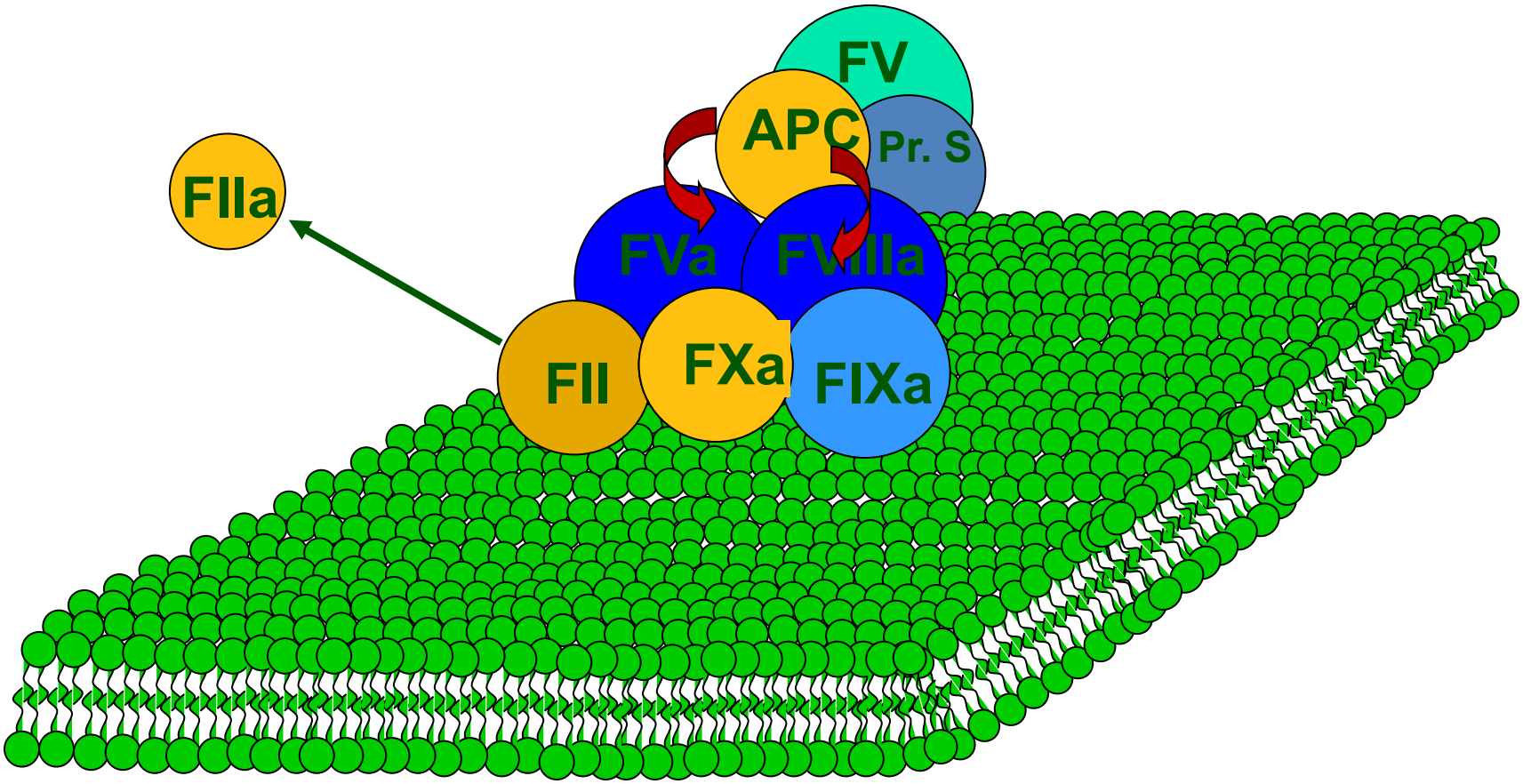
Hemostasis

Formation and regulation of FXa and thrombin - a challenging system with heterogenous catalysis!



Classification of hemophilia A and B as based on factor activity in plasma

Severe	<1 %
Moderate	1-5 %
Mild	>5 – 40 %

Blanchette VS et al; SSC/ISTH communication,
J Thromb Haemost 2014; 12, 1935-1939

Methods

One-stage APTT based

Two-stage clotting

Clot Waveform APTT-based

FIXa-driven Thrombin Generation

First and foremost:

Proper blood sampling and proper sample handling is a prerequisite to obtain reliable results!

”Garbage in = garbage out”

Principle of one-stage clotting method

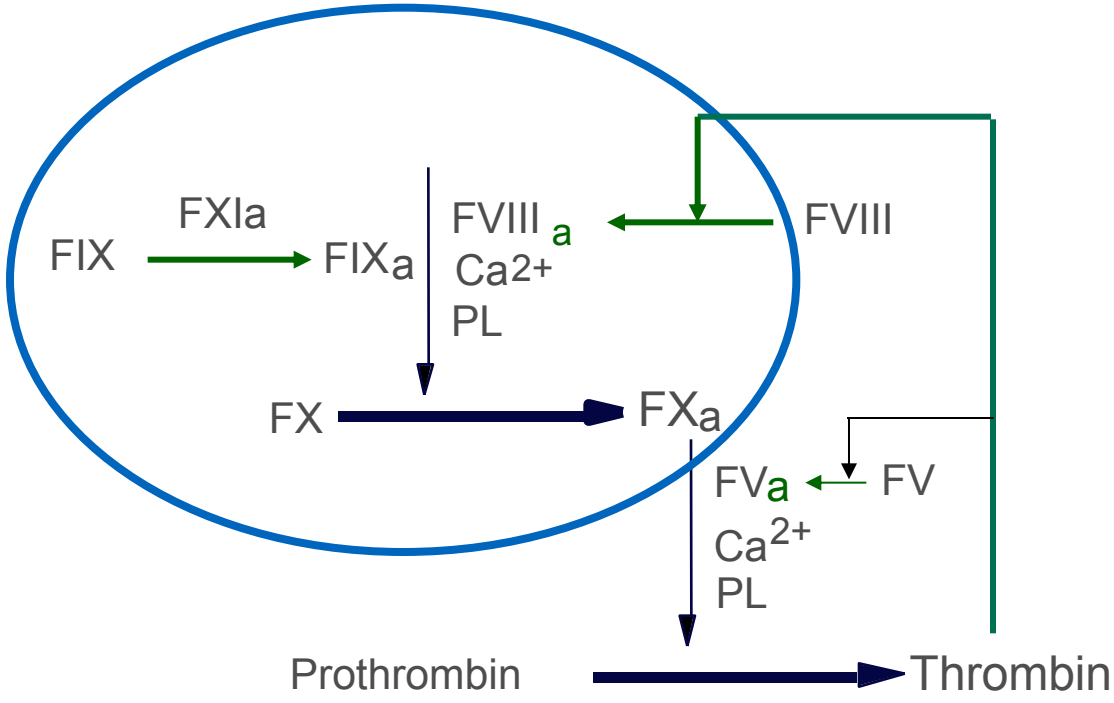
APTT-based method in which Factor Xa, thrombin and fibrin are generated in one single stage after an initial contact activation step for generation of Factor XIa.

1) Contact activator + phospholipids (APTT reagent)
   Sample plasma
   FVIII/FIX deficiency plasma (excess)
   → FXIa

2) Addition of calcium ions and recording of time for clot formation → FIXa, FVIIIa, FXa, thrombin and fibrin

Approach used in two-stage chromogenic FVIII/IX methods

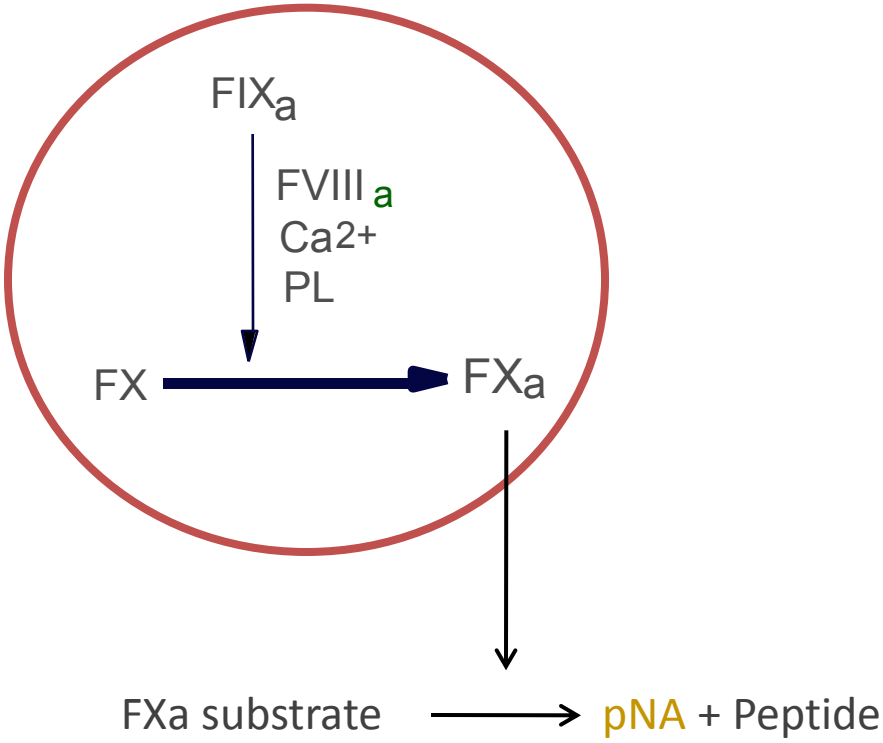
1) FXa generation with FVIII or FIX as rate limiting component



No use of FVIII/FIX deficient plasma

Approach used in two-stage chromogenic FVIII/IX methods

2) Determination of FXa from cleavage rate of a chromogenic FXa substrate



Publications on global methods reflecting factor activity

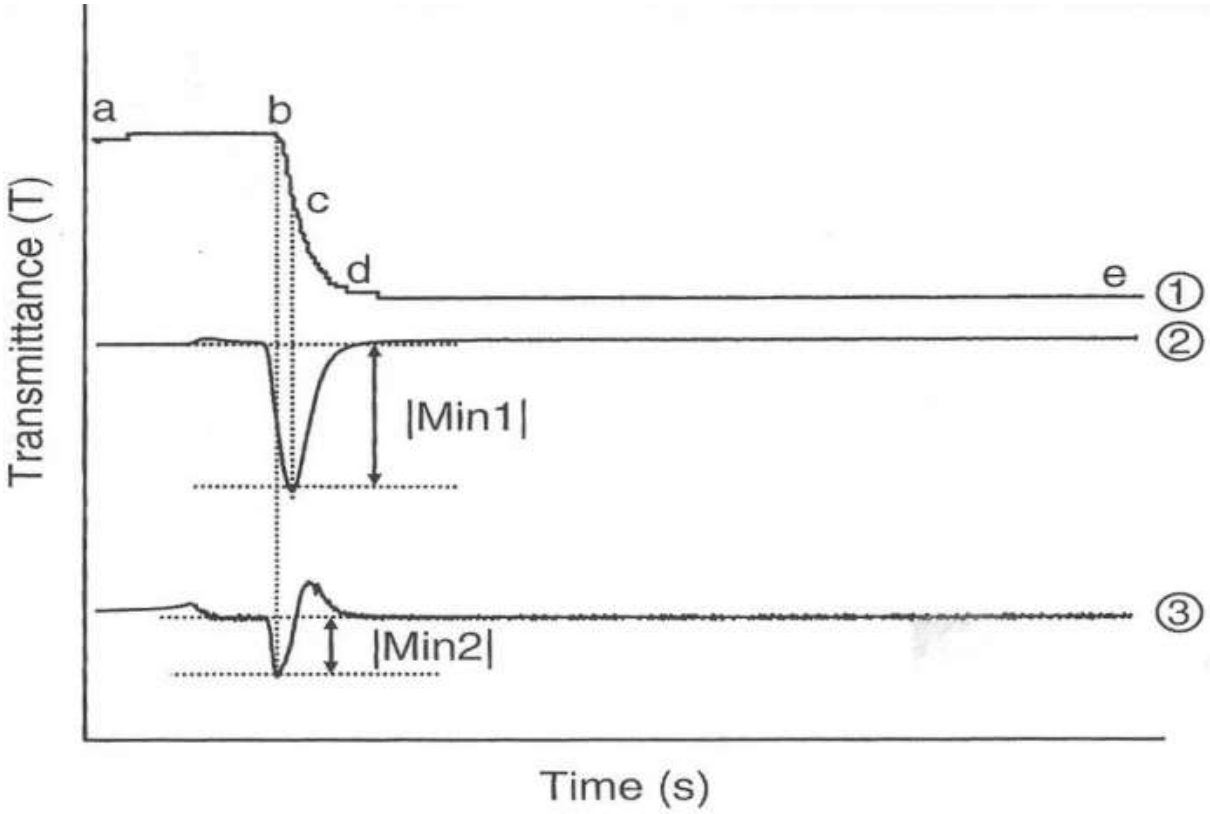
M Shima et al. *Thromb Haemost 2002; 87, 436-41.*

T Matsumoto et al. *J Thromb Haemost 2006; 4, 377-384.*

M Shima et al. "Towards standardization of clot waveform analysis and recommendations for its clinical applications." *J Thromb Haemost 2013; 11, 1417-20.*

M Ninivaggi et al. "Thrombin generation assay using factor IXa as trigger to quantify accurately factor VIII levels in haemophilia A." *J Thromb Haemost 2011; 9, 1549-55.*

Parameters determined in CWF method



From Matsumoto T et al. *J Thromb Haemost 2006; 4, 377-384*
Courtesy by Dr M Shima

Effect of various FIXa levels on thrombin generation in normal plasma

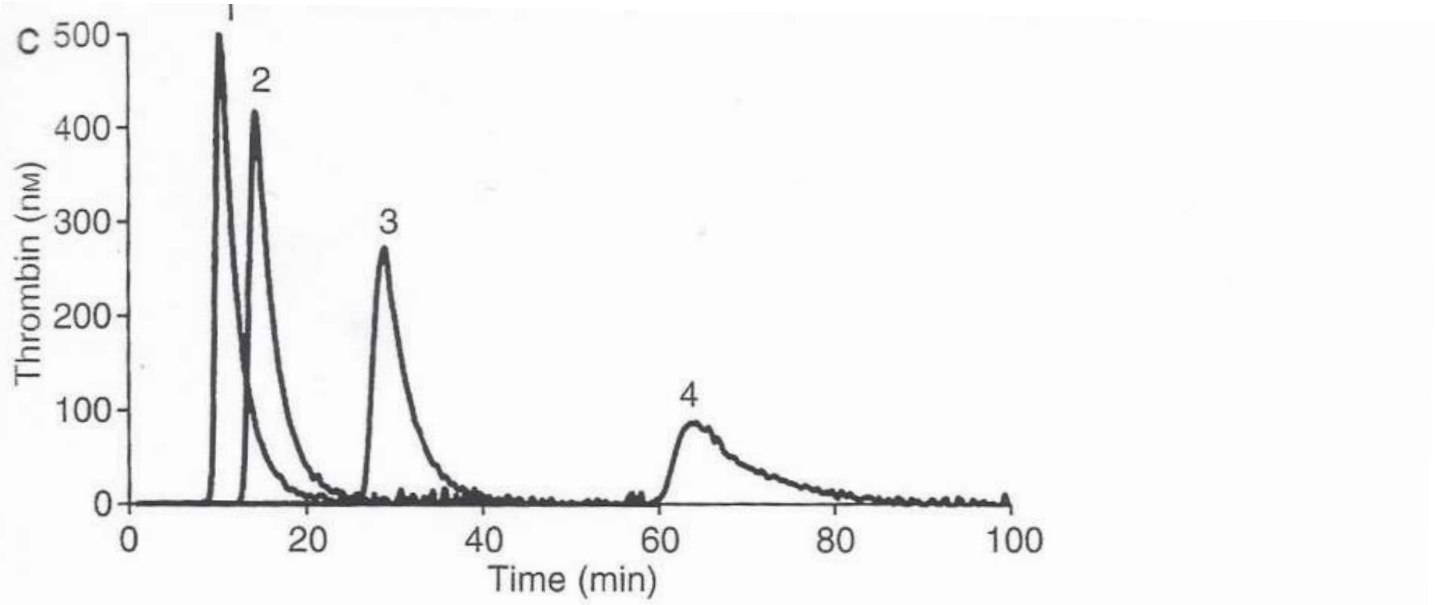


Fig. 1. Trigger dependency. (A, B) Thrombograms of TF (1:5, 2:2, 3:1 and 4:0.5 pM TF) triggered FVIII-deficient plasma (A) and normal plasma (B). (C) Thrombograms of FIXa (1:5, 2:1, 3:0.4 and 4:0.2 nM) triggered normal plasma. All experiments were performed in the presence of CTI (10 μg mL^{-1}) and phospholipids (4 μM).

Ninivaggi M et al. *J Thromb Haemost 2011; 8, 1549-1555*
Courtesy by Dr T Lindhout

Chromogenic FVIII kits

Product name	*Sample Diluent*	*Volume Plasma during activation of FX*
Coatest SP Factor VIII	Tris-1% BSA	0.31% (LR); 0.21% (HR)
Coamatic Factor VIII	Tris-1% BSA	0.62% (LR and HR)
Factor VIII Chromogenic	0.9% NaCl	1.1% (one range)
BIOPHEN FVIII:C	Tris-1% BSA	3.3% (LR); 0.81% (HR)
TECHNOCHROME FVIII:C	Imidazole, 0.2% BSA	3.3% (LR); 0.81% (HR)

Low ranges claim determination of 0.05 – 1% = 0.005 – 0.01 IU/mL

Reagents

Product name	*FX activation reagents* *Reagent 1*	*Reagent 2*	*Reagent 3*
Coatest SP FVIII	bFIXa, bFX, bFV, b**FII** FIIa generated in assay	Phospholipids	$CaCl_2$
Coamatic Factor VIII	bFIXa, bFX, b**FIIa** $CaCl_2$, phospholipids	None	None
Factor VIII Chromogenic	bFIXa, b**FIIa** $CaCl_2$, phospholipids	bFX	None
BIOPHEN FVIII:C	hFIXa, h**FIIa** $CaCl_2$, phospholipids	hFX	None
TECHNOCHROME FVIII:C	hFIXa, hFX, h**FIIa**, $CaCl_2$	Phospholipids	None

Chromogenic FIX kits

Currently two kits available

BIOPHEN Factor IX (Hyphen)	hFXIa, hFVIII, hFX, hFIIa
Rox Factor IX (Rossix)	hFXIa, hFVIII, hFX, bFV, hFII

Determination of low FVIII activities is never trivial with any method!

Large variation in results are regularly obtained in QC studies.

Example (n = 91 centers):	*Assigned*
Samples with FVIII **< 0.01** IU/mL	**0.01-0.05** IU/mL
One sample with **0.058** IU/mL	**0.015-0.36** IU/mL

(Preston FE et al. *J Thromb Haemost* 2003; 2, 271-274

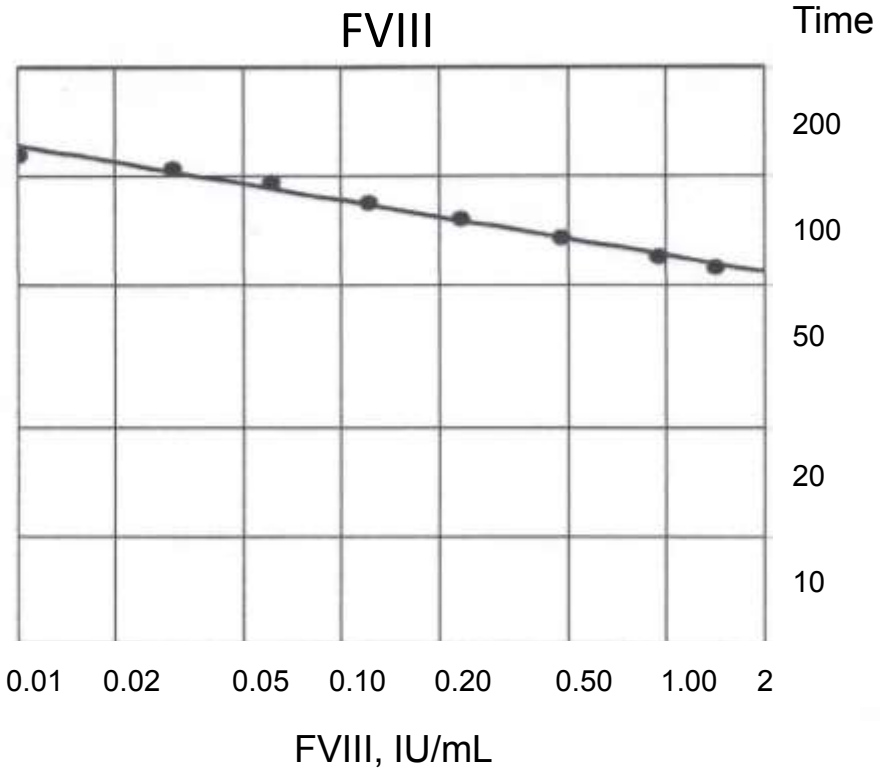
Influential factors in OS methods:
Choice of APTT reagent, phospholipid, buffer, FVIII deficiency plasma
See e.g. Barrowcliffe T. *Haemophilia* 2003; 9, 397-402
Butenas S et al. *Thromb Res* 2010; 126, 119-123

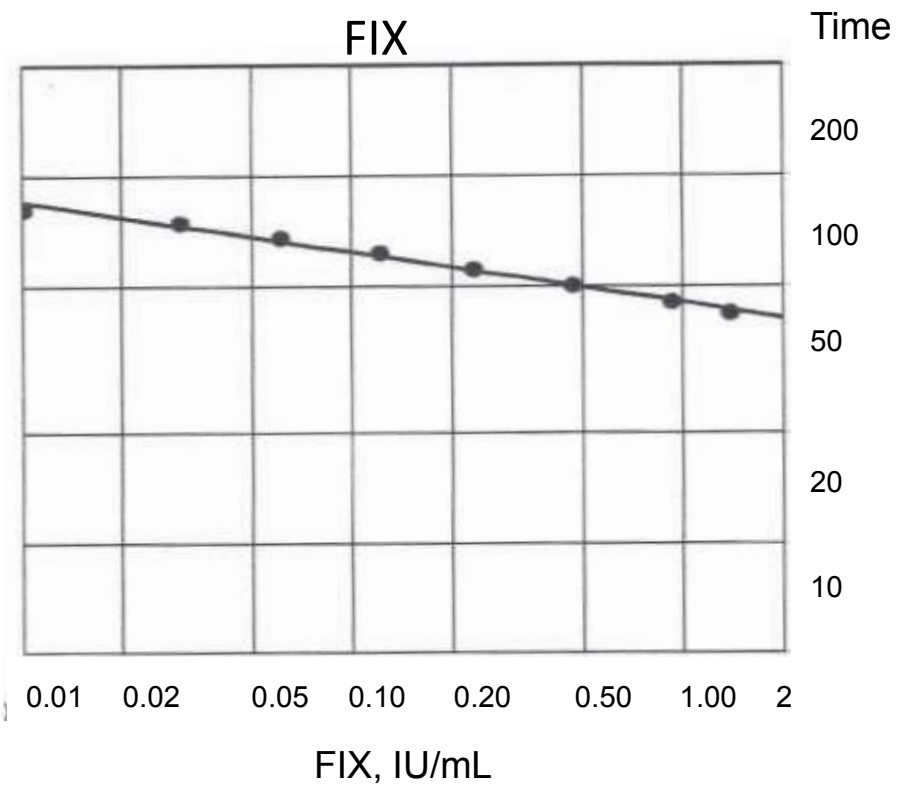
The one-stage clotting method can be improved by performing all dilutions of calibrator plasma in FVIII deficiency plasma. This may have a pronounced impact below 0.05 IU/mL
Cinotti S, Paladino E, Morfini M. *J Thromb Haemost* 2006; 4, 828-833.

For chromogenic methods, deficient plasma is not utilized. It is therefore crucial that manufacturers warrant a high lot-to-lot consistency with about overlapping calibration curves in the absence and presence of factor deficient plasma (constant plasma concentration)

Examples of dose-response curves for one-stage method

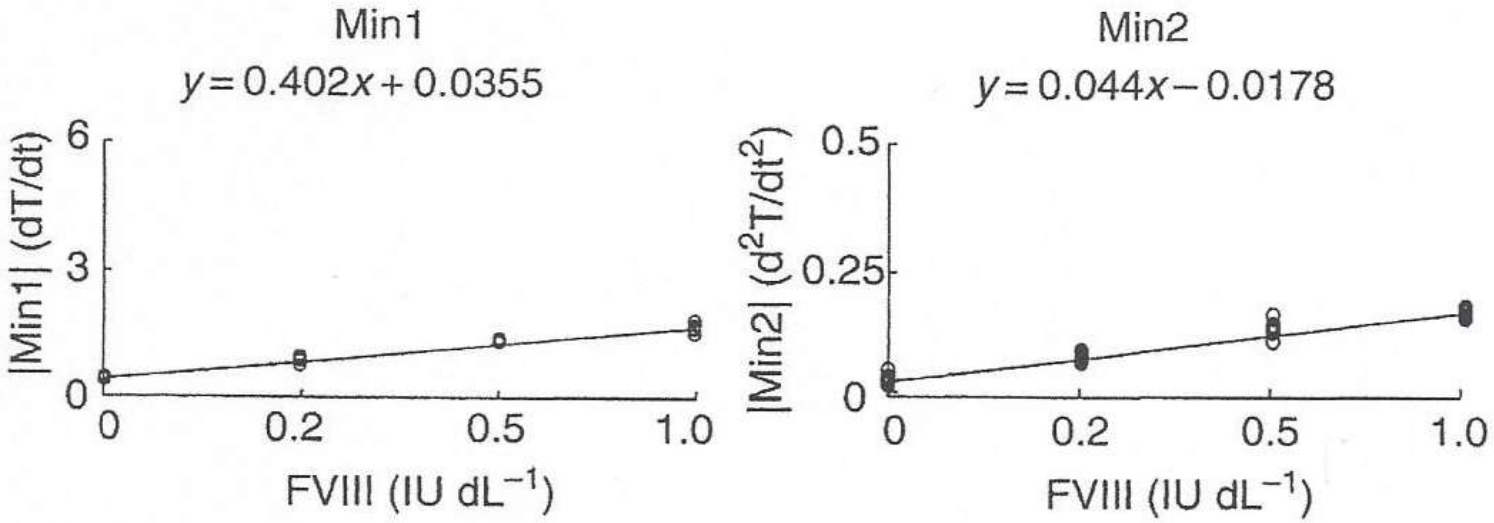
Instrument: Sysmex CS5100





Courtesy by Dr S Kitchen

CWF method: Resolution in 0 – 0.01 IU/mL range from spiking rFVIII into FVIII deficient plasma



From Matsumoto T et al. *J Thromb Haemost 2006; 4, 377-384*
Courtesy by Dr M Shima

CWF parameters: Mean results from 10 independent assays

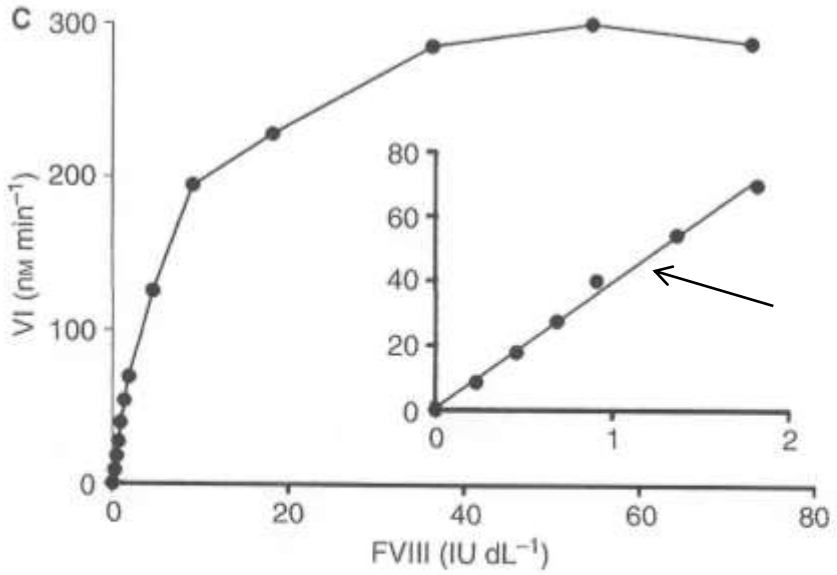
Table 2 Changes in waveform parameters at varying concentrations of FVIII and FIX

	0.0	0.2	0.5	1.0
Factor VIII (IU dL^{-1})				
Clot time (s)*	136.5	114.3	94.4	84.9
Min1 (%T s^{-1})*	0.4012	0.8723	1.2803	1.6037
Min2 (%T s^{-2})*	0.0264	0.0686	0.1200	0.1570
P-value†	← <0.01	→← <0.01	→← <0.01	→
Factor IX (IU dL^{-1})				
Clot time (s)*	114.8	95.5	84.7	76.9
Min1 (%T s^{-1})*	1.4645	2.2724	2.7634	3.2189
Min2 (%T s^{-2})*	0.1367	0.2210	0.2623	0.3022
P-value†	← <0.01	→← <0.01	→← <0.01	→

*$n = 10$.
†Student's *t*-test.
T, transmittance.

From Matsumoto T et al. *J Thromb Haemost 2006; 4, 377-384*
Courtesy by Dr M Shima

FIXa-triggered TG: Velocity index vs FVIII activity



Range 0 – 0.02 IU/mL

Fig. 3. Dependency of TG parameters on plasma FVIII activity. NPP was diluted with FVIII-deficient plasma to the indicated FVIII activities and activated with 5 nM FIXa and phospholipid (4 μM). Insets show plots of thrombogram parameters versus plasma FVIII levels between 0 and 2 IU dL^{-1}.

Ninivaggi M et al. *J Thromb Haemost 2011; 8, 1549-1555*
Courtesy b y Dr T Lindhout

FIXa-triggered TG: Effect of FVIII deficient plasma on assay accuracy

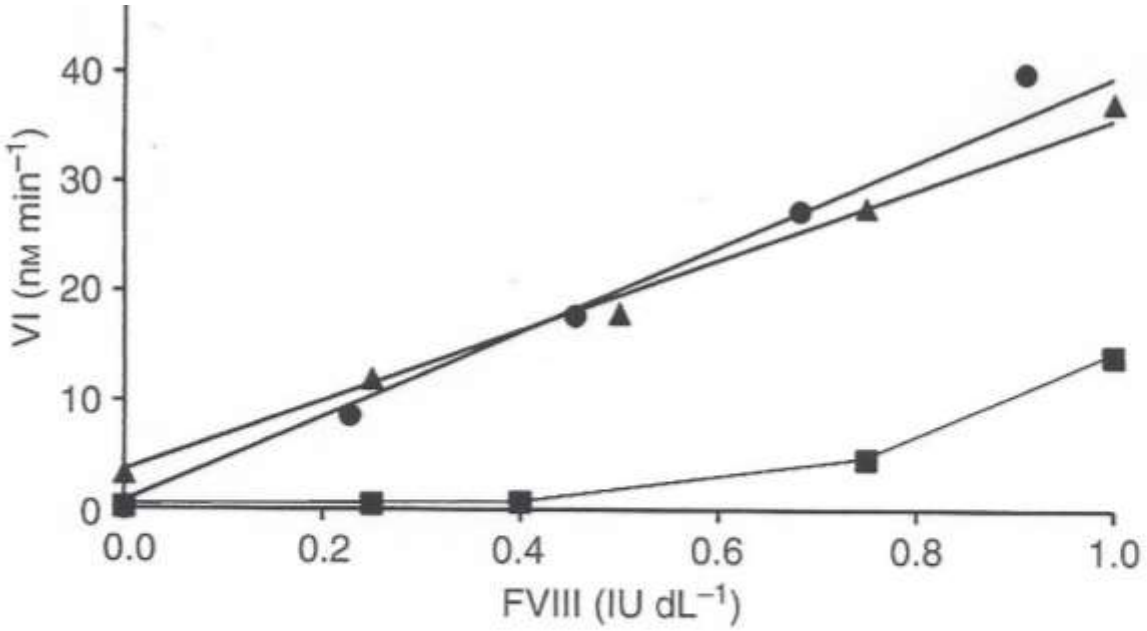


Fig. 4. Assay accuracy and FVIII-deficient plasma. Reference curves were prepared by diluting NPP in FVIII-depleted plasma A (●), FVIII-depleted plasma B (▲) and FVIII-depleted plasma C (■). Velocity index is plotted versus the FVIII activity.

Ninivaggi M et al. *J Thromb Haemost 2011; 8, 1549-1555*
Courtesy by Dr T Lindhout

Results with different chromogenic kits on calibration of FVIII Plasma Standards.

5th IS FVIII Plasma vs 4th IS Assigned potency *0.68 IU* (n = 40)

Results in IU/ampoule (No of labs)

	Coamatic FVIII	Coatest SP FVIII	Siemens	Immunochrome	Technochrom
Mean	0,70 (7)	0,66 (3)	0,80 (1)	0,68 (2)	0,67 (1)
CV	1,7%	4,4%		0%	

6th IS FVIII Plasma vs 5th IS Assigned potency *0.68 IU* (n = 52)

Results in IU/ampoule (No of labs)

	Coamatic FVIII	Coatest SP FVIII	Siemens	Immunochrome	Technochrom	Biophen
Mean	0,70 (7)	0,71 (5)	0,70 (2)	0,73 (1)	0,66 (1)	0,72 (4)
CV	2,3%	4,9%				2,4%

High agreement between clotting and chromogenic assays in NIBSC calibration of 4th IS Plasma II, VII, IX, X (09/172)

Table 1d: Ratios of clotting to chromogenic potency estimates for sample A relative to the 3rd IS

	Potency estimates (IU/ampoule)		Ratio Clotting/Chromogenic
	Clotting	*Chromogenic*	
FII	0.893 (n = 23)	0.889 (n = 3)	**1.004**
FVII	0.990 (n = 25)	0.965 (n = 3)	**1.026**
FIX	0.862 (n = 27)	0.888 (n = 1)	**0.971**
FX	0.886 (n = 23)	0.898 (n = 2)	**0.987**

Precision of RBD assays is similar to FVIII and IX assays and is related to degree of abnormality. CV% are shown.

Factor	5-10 IU/dl	10-20 IU/dl	20-30 IU/dl	30-60 IU/dl	60-130 IU/dl
VIII:C	52*	28*	19*	16	17
IX	50	17		16	14
II		21*	19*		10*
V					11
VII	39*		15*		10
X		17*	13*		9
XI			26*	22*	11

* Excludes > 5 SD outlier(s)

SSC Kyoto 2011

Courtesy by Dr S Kitchen

Assignment of FVIII activities in OS method from dilution of calibrator plasma with buffer vs FVIII deficiency plasma

APTT reagent: Pathromtin SL Buffer: Imidazole

FVIII U/dL	n	Method A Buffer	Method B VIII-DPL	Ratio A/B
< 1.0	22	0.55	0.05	≥ 9.5
1 - < 5	18	2.5	1.1	1.9 - 3.2
5 - < 10	10	7.1	4.2	1.8 - 2.2
10 - < 25	4	14.8	9.7	1.5 - 1.6
> 50	2	76, 102	75, 100	1.01

From Cinotti S, Paladino E, Morfini M. *J Thromb Haemost* 2006; 4, 828-833.

Extract of results from field study on N8 and Advate, spiked plasma samples

	Target **0.03** IU/mL		
	N8	Advate	SSC Standard
One-stage (n = 99)			
Mean	**0.047**	**0.046**	**0.84**
Range	0.013-0.113	0.013-0.105	0.56-1.20
Chromogenic (n = 15)			
Mean	**0.032**	**0.030**	**0.83**
Range	0.022-0.041	0.023-0.038	0.76-0.94

From Viuff D et al. Haemophilia 2011; 4, 695-702

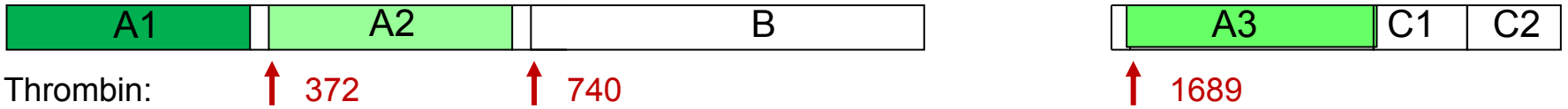
Example of resolution with a chromogenic FIX method (Rox Factor IX)

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

Bryngelhed P, Rosén P, Rosén S. Poster TH208 at ISTH Kyoto 2011

Diagnosis of hemophilia – FVIII mutations related to discrepancy between one-stage, two-stage and chromogenic methods



Mutations in the A1, A2 and A3 domains may result in OS > TS, CS

Denson KWE, Biggs R, In *Human Blood Coagulation, Haemostasis and Thrombosis* 1976; 310-364. Ed.: Biggs R
Hathaway WE et al, *Thromb Haemost* 1983; 50, 357
Duncan EM et al, *Br J Haematol* 1994; 87, 846-848.
Rudzki Z et al, *Br J Haematol* 1996; 94, 400-406.
Pipe SW et al, Blood 1999; 93, 176-183
Schwaab R et al, *Br J Haematol* 2000; 109, 523-528.

Mutations in the thrombin cleavage region sites may result in OS < CS

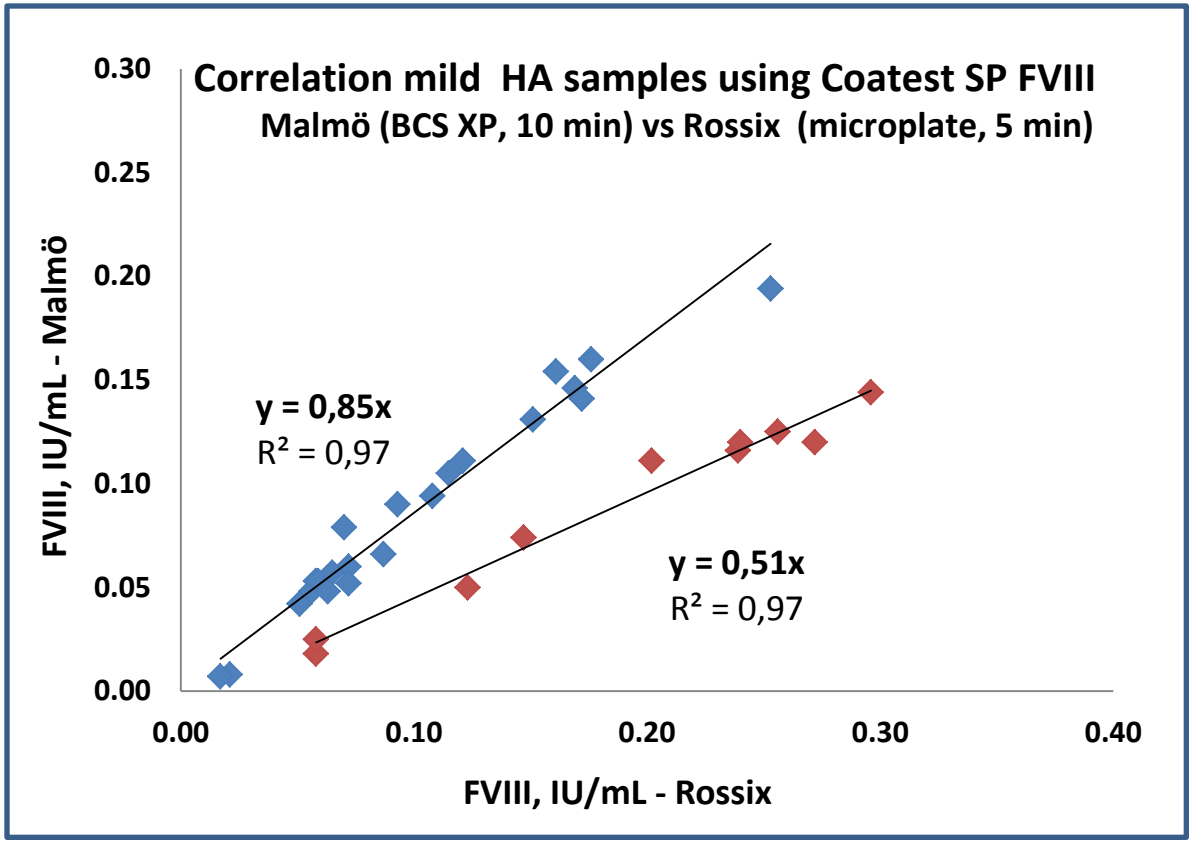
Gitshier J et al, *Blood* 1988, 72, 1022-1088
Goodeve AC et al, *Thromb Haemost (Suppl)* 2001; Abstract P1370
Mumford AD et al, *Thromb Haemost (Suppl)* 2001; Abstract P2861
Brondke H, Herbiniaux U, Oldenburg J. *J Thromb Haemost* 2009; 7, Supplement 2: AS-WE-002

Recent paper with broad coverage:

Pavlova A et al, *Thromb Haemost* 2014; 111: 851-861:

Mild hemophilia – Discrepancy even using the same chromogenic kit Coatest SP FVIII but with different incubation times

Sample No	Mutation	Sample No	Mutation
1	R1941Q	5	R527W
2	Y1680F	7	R527W
3	Y1680F	9	R527W
4	T295A	16	R527W
6	Y1680F	18	R531H
8	Y1680F	19	V663A
10	L1756V	20	R527W
11	Y1680F	21	R527W
13	Y1680F	23	G479R
14	Y1680F	33	R531C
15	T275I		
17	T295A		
24	T295A		
22	T295A		
26	T295A		
27	T295A		
28	L412F		
29	Y1680F		
30	Y1680F		
31	M702L		
32	F2101L		
34	F2101L		



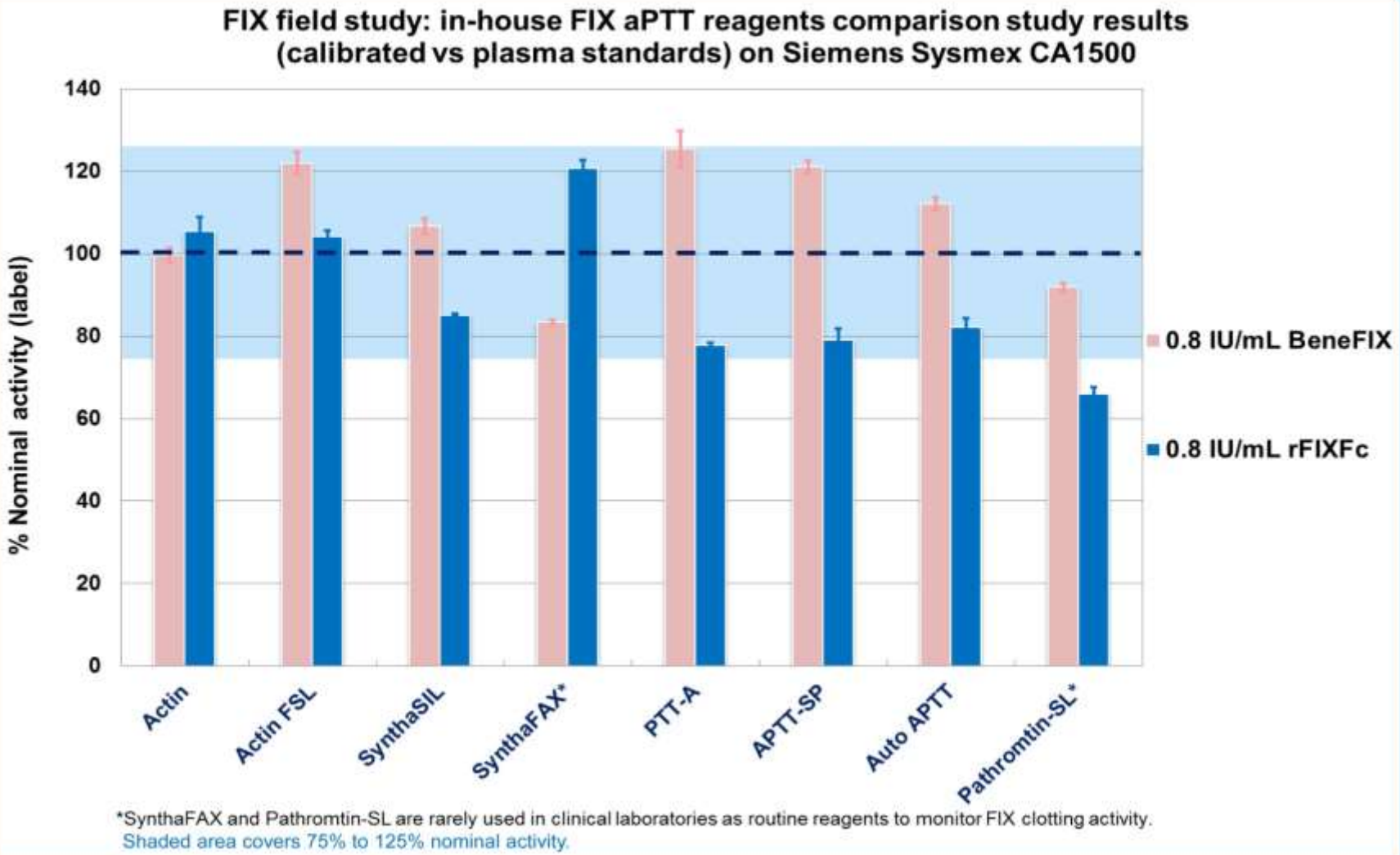
Activated R527W, and presumably also R531H, V663A, G479R and R531C, has a shorter t1/2 than wild-type FVIII!

It is highly recommended to perform diagnosis of hemophilia with both one-stage and two-stage (chromogenic) methods.

New, so called long-life, rFVIII and rFIX:

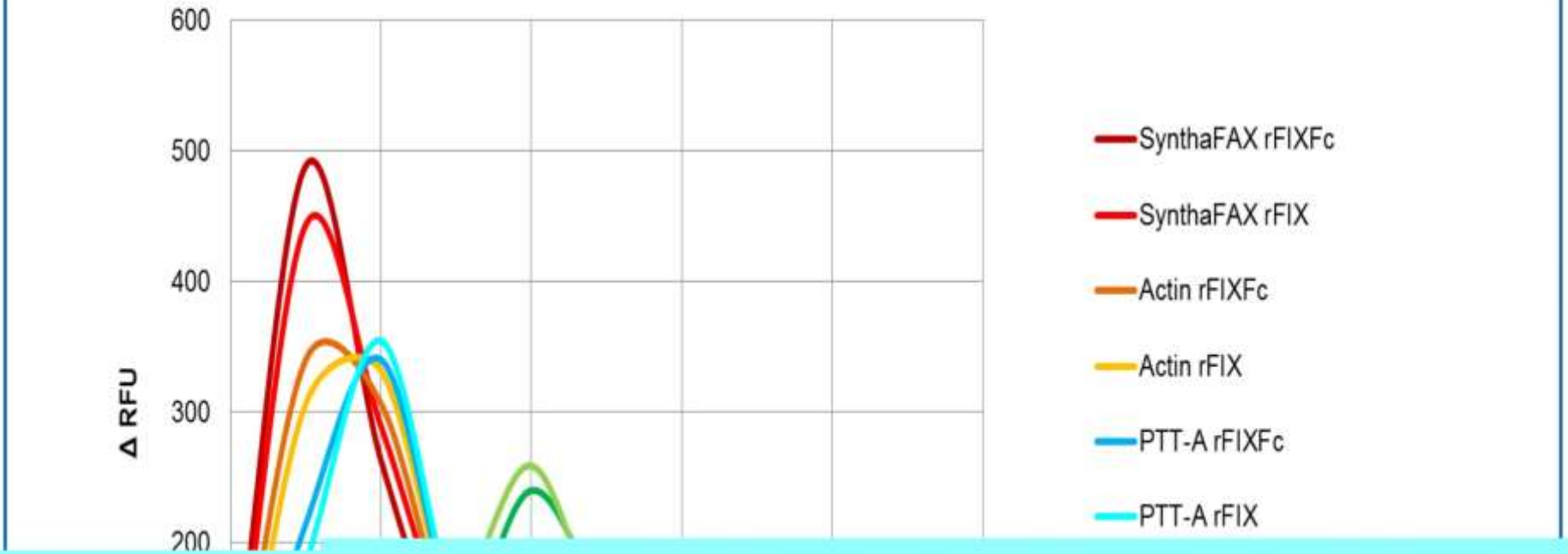
Increased complexity in potency assignments, which may well translate into clinical coagulation laboratories

Figure 1. In-house evaluation of rFIXFc and BeneFIX ativity with different aPTT reagents [1]



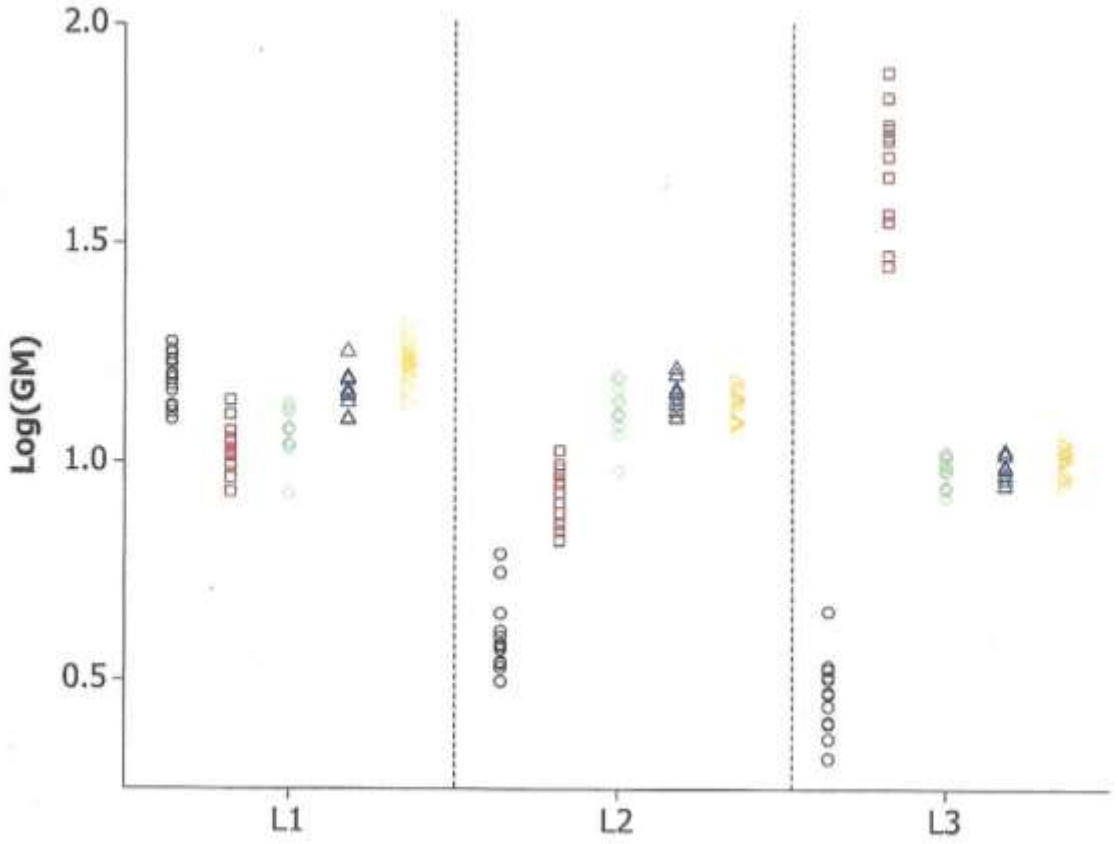
Courtesy by Dr J Sommer

Figure 6. Monitoring thrombin generation during the calcium-dependent stage of aPTT with different aPTT reagents



Courtesy by Dr J Sommer

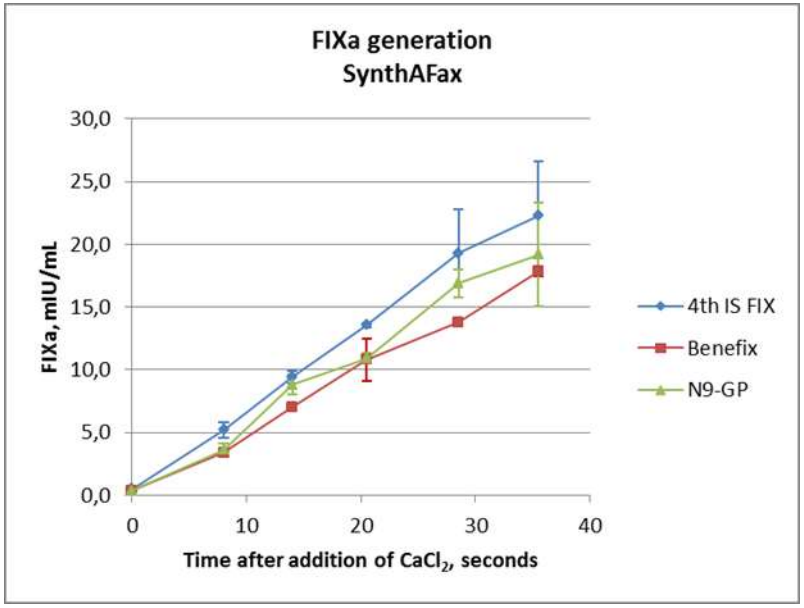
Potency assignments of rFIX concentrates vs 4th IS 07/182. NIBSC Multicenter study 2013

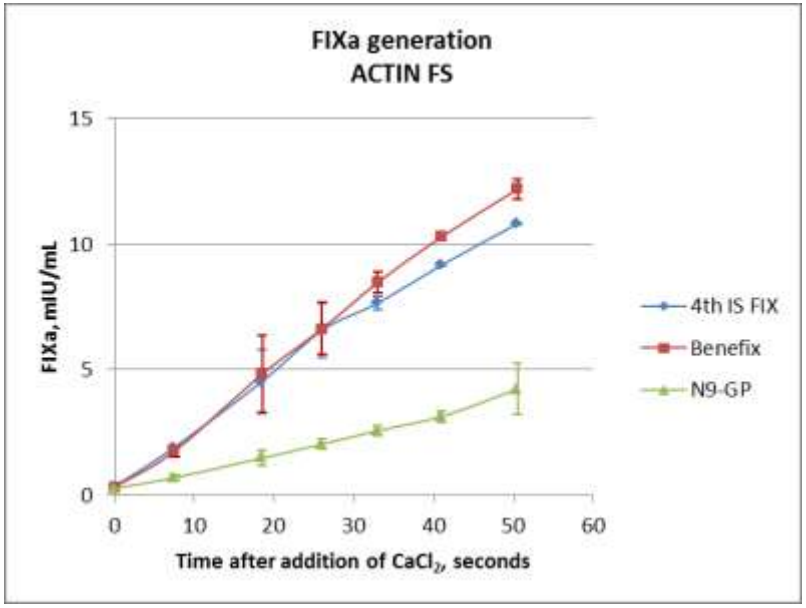


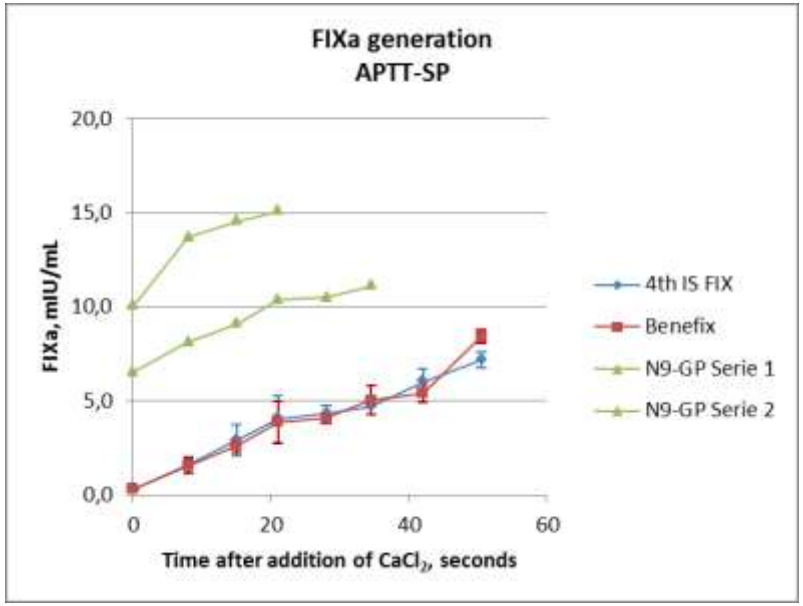
	L3 Mean, IU/mL
Actin FS	**2.9**
APTT SP	**47.5**
SynthAFax	**10.1**
Hyphen	**9.4**
ROX	**9.7**

Courtesy by Dr E Gray

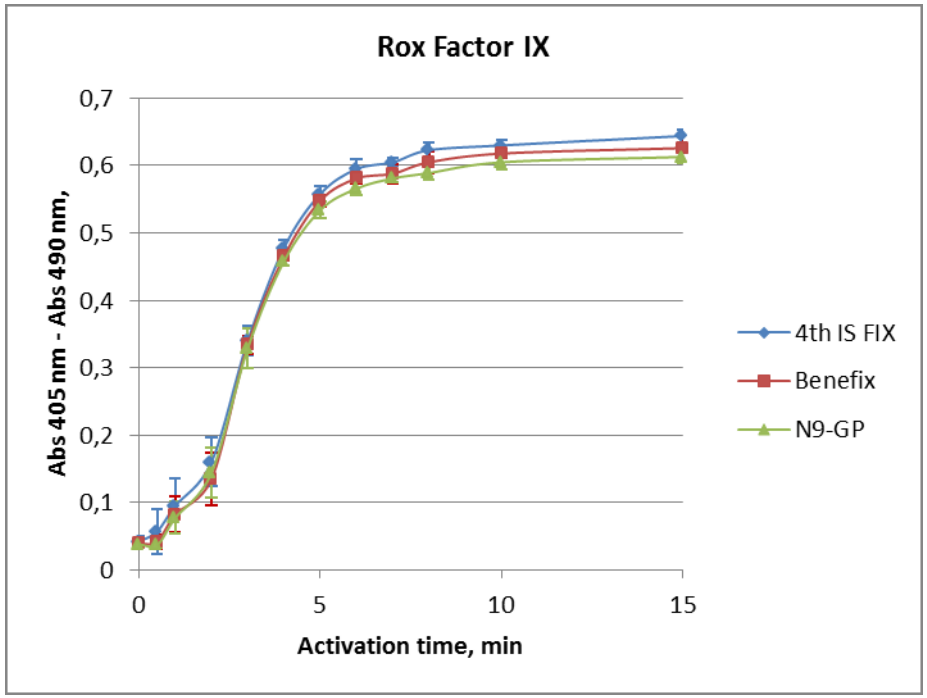
Subsampling after addition of $CaCl_2$ – FIXa generation



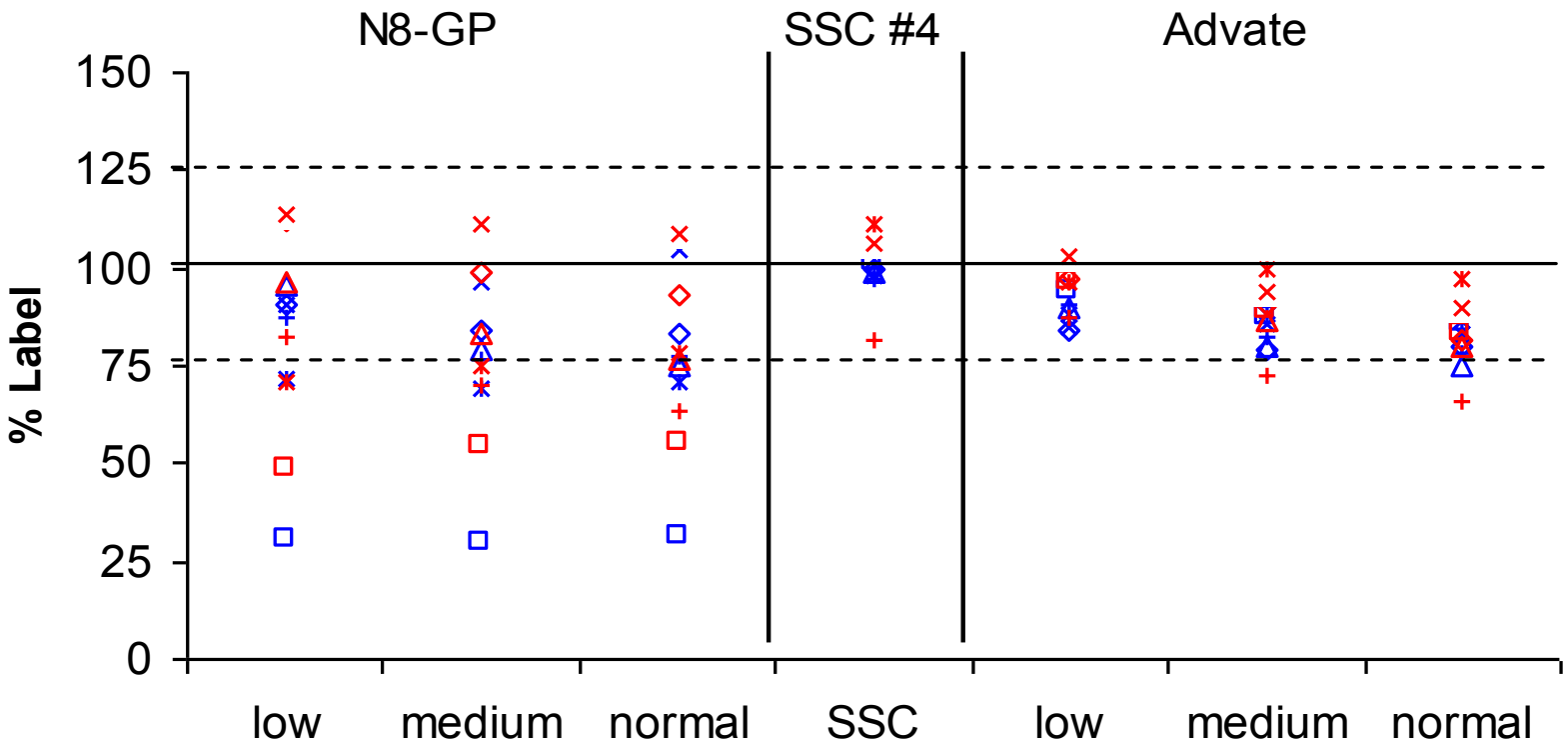




Activation kinetics – Rox Factor IX



Recovery of FVIII activity vs labelled value with OS methods after spiking of N8-GP and Advate into FVIII deficient plasma



Three samples spiked with N8-GP and Advate. APTT reagents: Actin FS (◊), APTT SP (□), DG Synth (Δ), Pathromtin (×), SynthASil (∗) and SynthAFax (+).
Results from Sheffield in blue and from Malmö in red.
Bowyer AE, Hillarp A et al. Poster SSC Milwaukee 2014
Courtesy by Dr Hillarp

Will the species of FIXa and FX be of importance in FVIII activity determinations of new long-life rFVIII?

From SSC 2004:
FVIII potency assignment vs 6th IS with all bovine and all human species of FIXa and FX and of thrombin or prothrombin + FV

Sample	Bovine, FII +FV		Human, FII + FV		Bovine, FIIa		Human, FIIa	
	IU/mL	CV,%	IU/mL	CV,%	IU/mL	CV,%	IU/mL	CV,%
Octonativ-M	***41***	4.6	***40***	8.1	***41***	4.6	***42***	8.8
Recombinate	***86***	8.1	***88***	4.3	***87***	4.3	***92***	6.7
ReFacto	***83***	8.0	***81***	7.7	***87***	1.1	***87***	5.2

r_{mean}= 0.998 (range 0.991-1.00); n = 58

We will learn, possibly during 2015

Conclusions

Measuring low FVIII and FIX activity requires strict adherence to proper blood sampling and handling

Chromogenic methods seem to provide a higher resolution and accuracy at low factor levels than regular one-stage methods

One-stage methods will show improved accuracy by preparing calibrator dilutions in a constant amount of deficient plasma

The APTT clot waveform method shows promise of proper discrimination even below 0.01 IU/mL

Using FIXa as trigger in the thrombin generation test seems to transform this test into a very sensitive method for determining FVIII activity even below 0.01 IU/mL

New generation of rFVIII/rFIX therapeutics with a prolonged half-life brings increased complexity in factor activity determinations