Recommendations for Laboratory Measurement of FVIII and FIX type I inhibitors

Piet Meijer
ECAT Foundation
The Netherlands

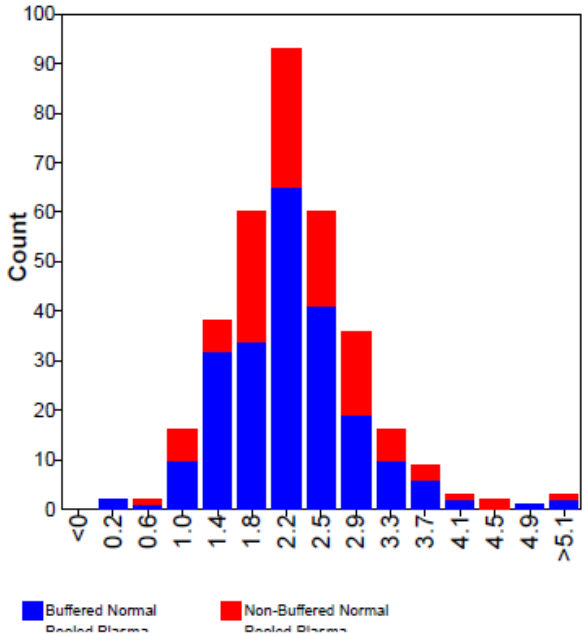
Introduction

- The major complication of haemophilia therapy today is the development of anti-drug (anti-factor) antibodies termed inhibitors.
- In severe haemophilia A, FVIII inhibitors form in approximately 30% of patients, usually during the first 20–30 days of CFC exposure.
- In severe haemophilia B, the cumulative incidence of FIX inhibitor development is lower than in severe haemophilia A and is as high as 4–5% after a median of only 9–11 exposure days.
- Detection and monitoring of FVIII and FIX inhibitors are essential to patient management.

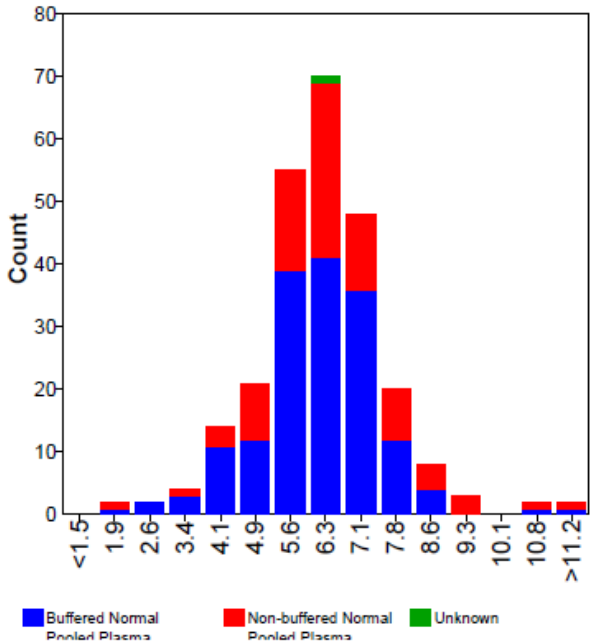
Introduction

- The assay methodology for FVIII and FIX inhibitors is rather complex and a number of variables may influence the test result.
- The complexity of the assay system may result in undesirably high within- and between-laboratory variation in test results.
- Considerable between-laboratory variation for FVIII inhibitor testing (30 – 60%) has been shown by several providers of external quality assessment programs.
- These observations demonstrate the lack of standardization for FVIII and FIX inhibitor testing.

FVIII Inhibitor



FIX Inhibitor



	N	Mean (BU/mL)	Range (BU/mL)	CV (%)
Non-buffered NPP	116	2.3	0.7 – 5.2	**30.4**
Buffered NPP	225	2.1	0.0 – 8.4	**31.1**

	N	Mean (BU/mL)	Range (BU/mL)	CV (%)
Non-buffered NPP	87	6.2	2.2 – 11.7	**18.5**
Buffered NPP	163	6.3	1.8 – 13.0	**19.0**

ICSH INTERNATIONAL COUNCIL FOR STANDARDIZATION IN HAEMATOLOGY

Piet Meijer[1], Flora Peyvandi[2], Guy Young[3], Rajiv Pruthi[4], Silmara de Lima Montalvão[5], Steve Kitchen[6]

1) ECAT Foundation, Voorschoten, The Netherlands
2) Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Angelo Bianchi Bonomi Hemophilia and Thrombosis Center, Milan, Italy ; Università degli Studi di Milano, Department of Pathophysiology and Transplantation, Milan, Italy
3) Children's Hospital Los Angeles, University of Southern California Keck School of Medicine, Los Angeles, CA, USA
4) Division of Hematology and Hematopathology, Mayo Clinic, Rochester, MN 55905, USA.
5) Laboratory Hemostasis, Hematology and Hemotherapy Center, University of Campinas UNICAMP, Campinas-SP, Brazil.
6) Sheffield Haemophilia and Thrombosis Centre, Sheffield United Kingdom

Scope

Guidance on a standardised procedure for the laboratory measurement of factor VIII (FVIII) and factor IX (FIX) type I inhibitors.

Outline of the guidance document

Introduction

Type I and type II inhibitors

Scope of the recommendations

Screening Inhibitors

- *APTT Mixing Test*
- *Immunological testing*

Assay Principle

Sample requirements

- *General*
- *Pre-analytical heat treatment*
- *Sample dilution*

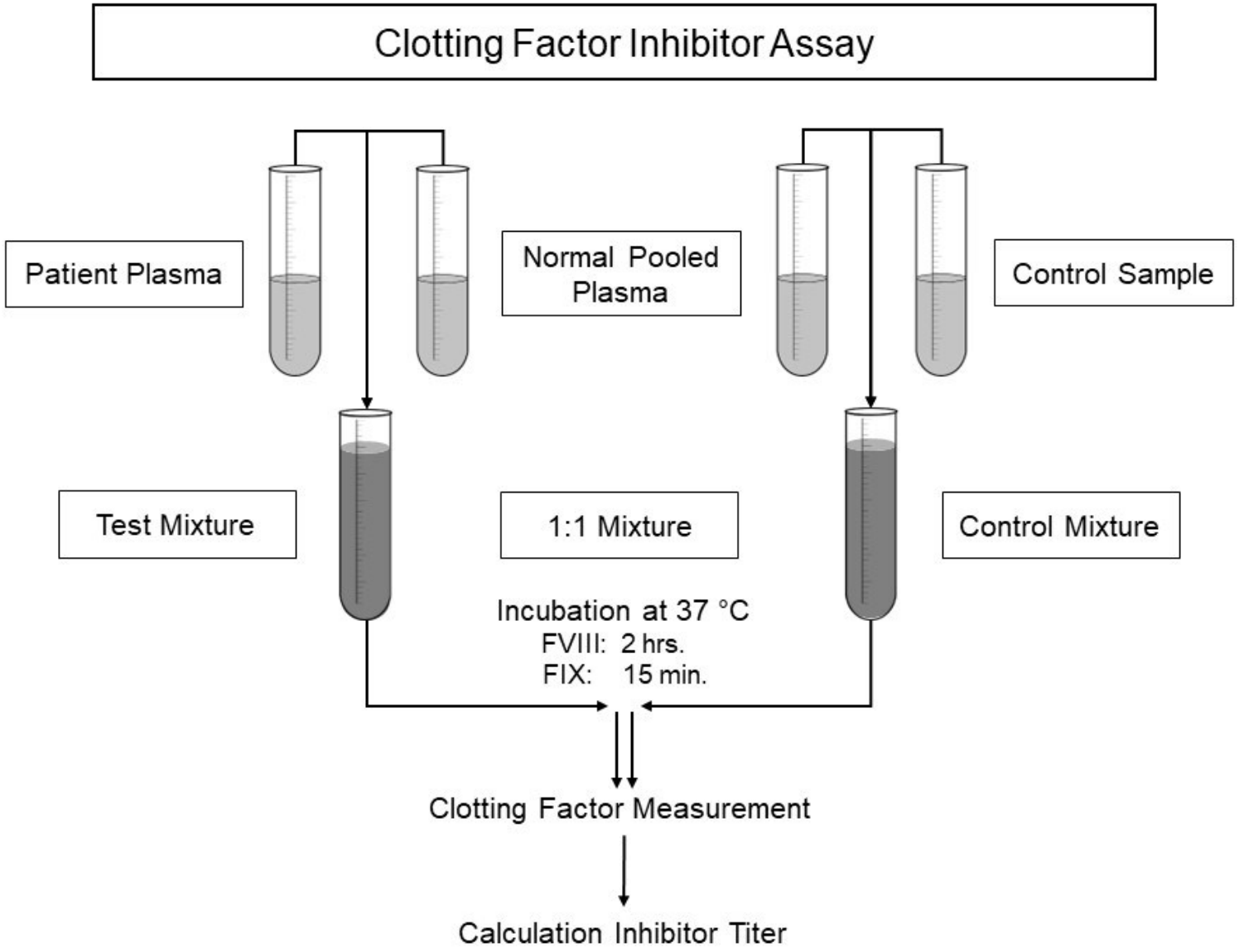
Testing requirements and interpretation

- *Normal pooled plasma*
- *Buffering normal pooled plasma*
- *Control Mixture*
- *Mixture*
- *Incubation time and temperature*
- *Residual Factor VIII or FIX measurement*
- *Calculation of the inhibitor titre*
- *Assay positivity*

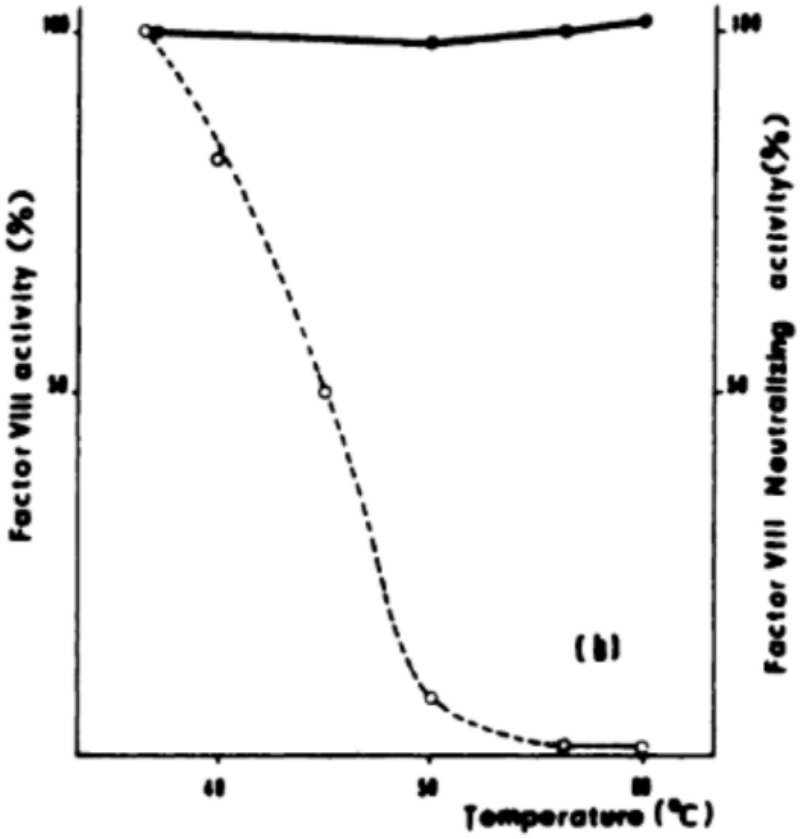
Quality Assurance

Interferences

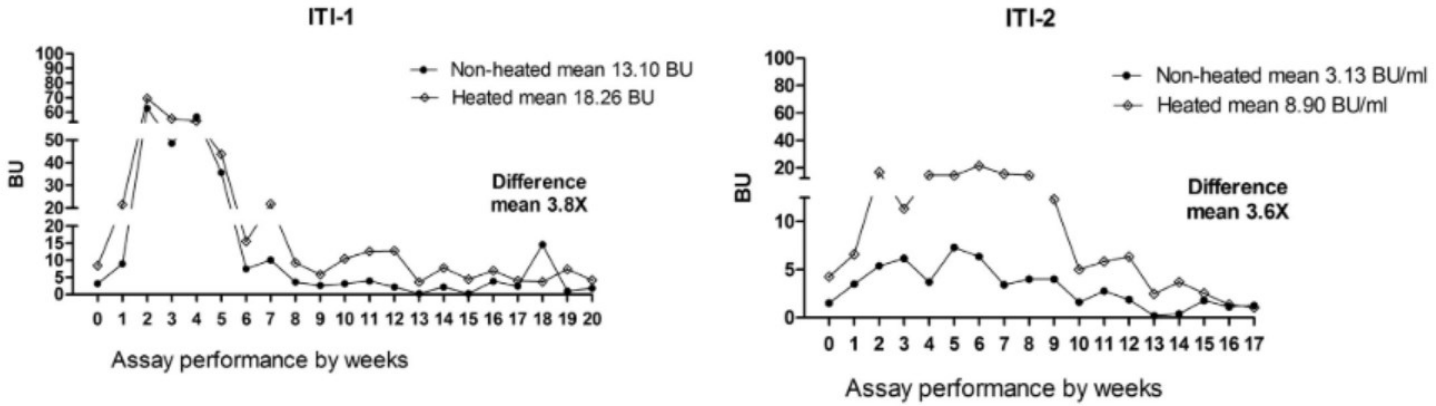
- *Anticoagulation therapy*
- *Lupus Anticoagulants*
- Other coagulation inhibitors
- Emicizumab (bispecific antibodies)
- Other causes


Clotting Factor Inhibitor Assay
Patient Plasma
Normal Pooled Plasma
Control Sample
Test Mixture
1:1 Mixture
Control Mixture
Incubation at 37 °C
FVIII: 2 hrs.
FIX: 15 min.
Clotting Factor Measurement
Calculation Inhibitor Titer

Sample requirements: pre-heat treatment

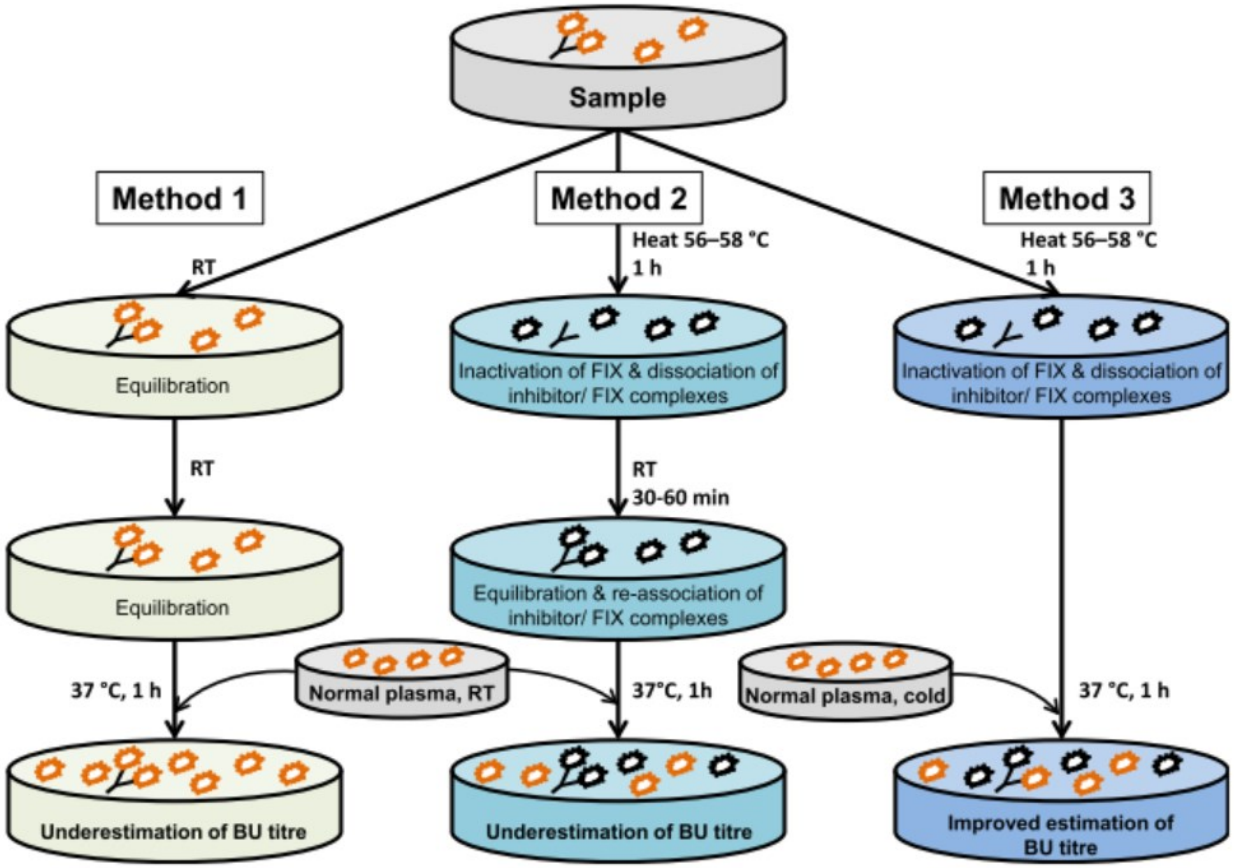


Allain, J.P. *et al* Blood, 1973; 42: 437-44.



de Lima Montalvao, *et al* Thromb Res, 2015; 136: 1280-4.

Sample requirements: pre-heat treatment



Conclusion:
Heat pretreatment is recommended for detecting FIX inhibitors in samples with residual FIX:C. The heat/cold modification improved the sensitivity of the Nijmegen-Bethesda assay, resulting in higher tolerance for residual FIX:C.

Millner, A.H. *et al* Int J Lab Hematol, 2016; 38: 639-647.

Sample requirements: sample dilution

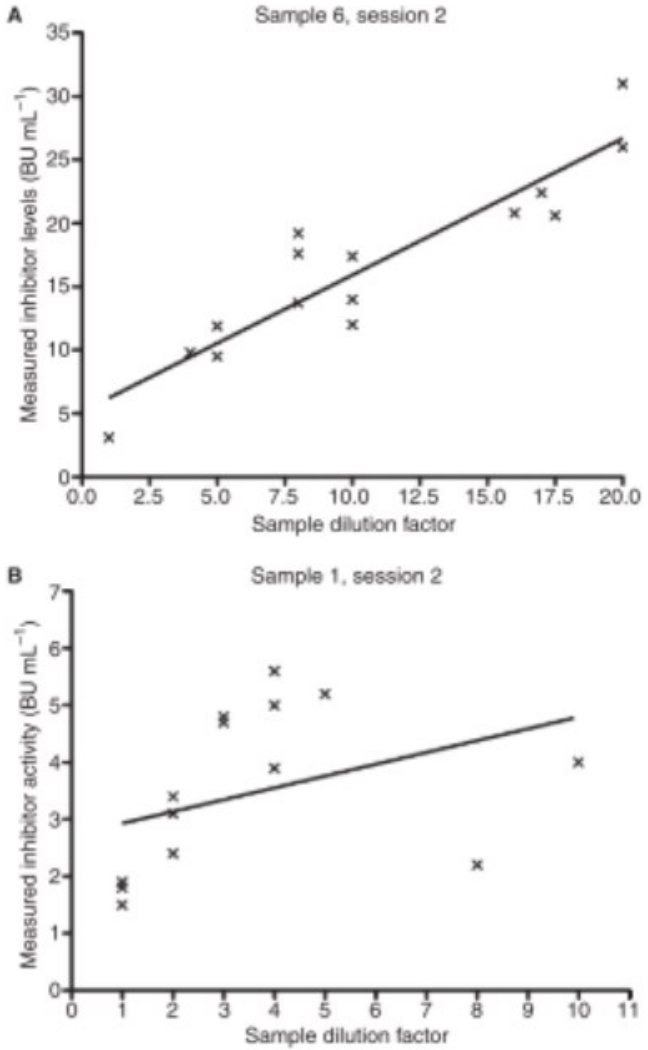


Fig. 3. Relationship between inhibitor activities and sample predilution factors of samples six (A) and one (B) measured by the participating laboratories in session 2.

Verbruggen *et al* J Thromb Haemost, 2011; 9: 2003-8.

Sample requirements: sample dilution

Expected Inhibitor range (BU/ml)	Corresponding dilution factor
0 - 2.0	Neat (undiluted)
2.0 - 8.0	4 (= 1+3)
5.0 - 20.0	10 (= 1+9)
12.5 - 50.0	25 (=1+24)
25.0 – 100.0	50 (=1+49)

Effect standardisation dilution factors

	Sample 1	Sample 2
	9.5 BU/mL	1.0 BU/mL
Overall	33.6%	36.9%
Recommended dilution factors	27.8%	30.0%
Suboptimal dilution factors	35.1%	46.7%

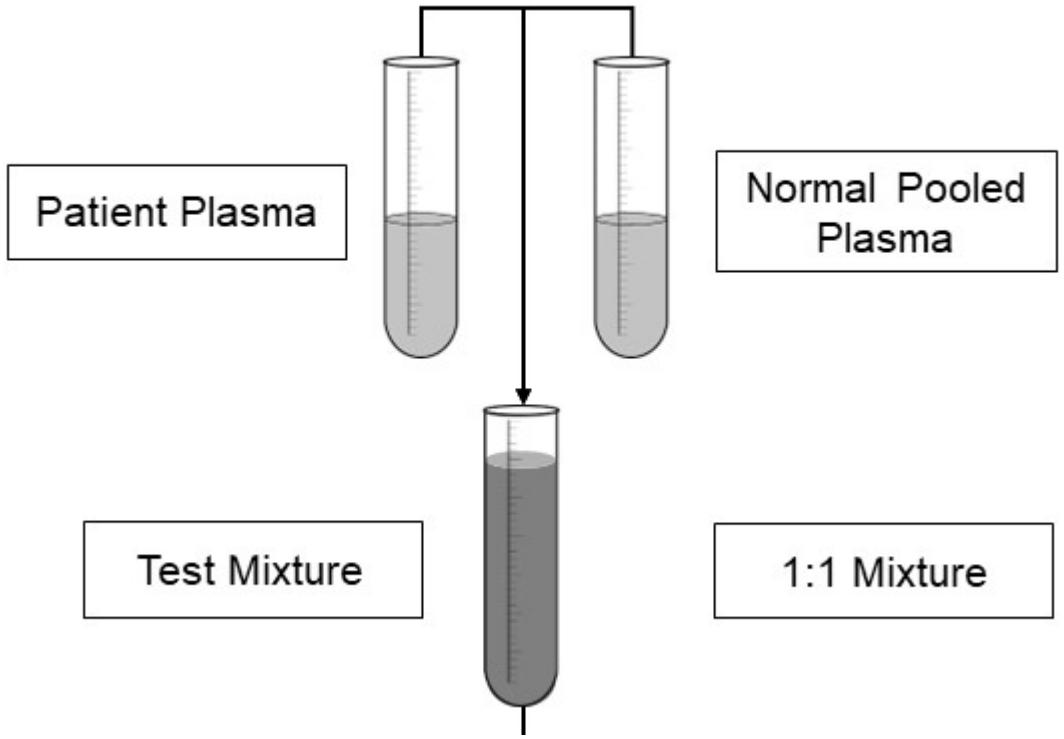
ICSH INTERNATIONAL COUNCIL FOR STANDARDIZATION IN HAEMATOLOGY

Consensus recommendations on sample requirements

1. Samples, both the clinical samples and the control plasma, should be preheated for 30 minutes at 56°C, followed by a centrifugation step for 2 minutes at 4000 x g.

2. Unknown samples should be measured in a series of dilutions. Samples with an inhibitor level in a known range should be measured with a fixed dilution factor.

Testing requirements and interpretation: normal pooled plasma



The basic principle of an inhibitor test is to mix the patient sample 1:1 with a normal pooled plasma.

A pooled plasma should be used to ensure that the level of FVIII or FIX is close to 1 IU/mL (= 100%).

A lower factor level in the normal pooled plasma may result in the overestimation of the inhibitor titre, while a higher factor level may give an underestimation of the inhibitor titre.

Testing requirements and interpretation: normal pooled plasma

Simulation of the effect of the clotting factor level in NPP

NPP	Sample	Control	%RA	Titer
110%	30	55	54.5	0.88
100%	**25**	**50**	**50**	**1**
90%	20	45	44.4	1.17
80%	15	40	37.5	1.42

Testing requirements and interpretation: buffering normal pooled plasma

Table 1 pH values and remaining FVIII : C activities in test- and control mixtures of the original and modified Bethesda assay

Assay mixtures	pH before incubation	pH after 2 h incubation	Remaining FVIII : C activity (%)
Non-buffered N-pool and haemophilic plasma (inhibitor free) 1 : 1	7.7 ± 0.1	8.3 ± 0.1	68 ± 6
Non-buffered N-pool and 0.1 M imidazole buffer pH 7.4 1 : 1	7.6 ± 0.1	7.8 ± 0.1	83 ± 6
0.1 M imidazole buffered N-pool pH 7.4 and haemophilic plasma 1 : 1	7.4 ± 0.1	7.4 ± 0.1	95 ± 5
0.1 M imidazole buffered N-pool and immunodepleted factor VIII deficient plasma 1 : 1	7.4 ± 0.1	7.4 ± 0.1	97 ± 4
0.1 M imidazole buffered N-pool and 0.9% saline 1 : 1	7.4 ± 0.1	7.4 ± 0.1	83 ± 8

All data are the means of 5 determinations ± the standard deviation.

Verbruggen *et al.* Thromb Haemost, 1995; 73: 247-51

Testing requirements and interpretation: buffering normal pooled plasma

Buffer	
Imidazole	Mix 1 volume of 4M Imidazole buffer with 39 volumes of normal pooled plasma. After mixing, the pH of the buffered normal pooled plasma should be verified and eventually adjusted between 7.3 and 7.5.
Hepes	Mix 1 volume of 1M Hepes buffer with 9 volumes of normal pooled plasma. After mixing, the pH of the buffered normal pooled plasma should be verified and eventually adjusted between 7.3 and 7.5.

It is demonstrated that Hepes buffer gives a higher change in the FVIII activity than imidazole buffer.

Miller, C.H. *et al* Haemophilia, 2018; 24: e116-e119

Testing requirements and interpretation: control mixture

Bethesda Assay	▶	Nijmegen Assay
Imidazole buffer	▶	Factor deficient plasma

Reason: comparable protein concentrations in both the assay and control mixture.

Testing requirements and interpretation: control mixture

Control Mixture (50/50 with BNPP)	Mean Inhibitor Titre NBU/ml (S.D) n=16
4% BSA	1.59 (0.12)
4% BSA + vWF (0.6 IU/ml)	1.63 (0.16)
Congenital FVIII :C Deficient Plasma	1.61 (0.12)

BNPP = Buffered Normal Pool Plasma ; S.D. = Standard Deviation

	Inhibitor Titre (NBU/ml) Control Mixture (50/50 with BNPP)		
FVIII:C Inhibitor Patient	4% BSA	Congenital FVIII:C Deficient	Chemically Depleted FVIII:C Deficient
AA54	0.45	0.85	0.50
NN024	0.7	nd	O.55
AA56	0.9	1.00	0.48
LL33	1.05	1.15	0.98
WW41	1.38	1.50	1.33
L503	1.68	1.48	1.73

n.d. = not determined

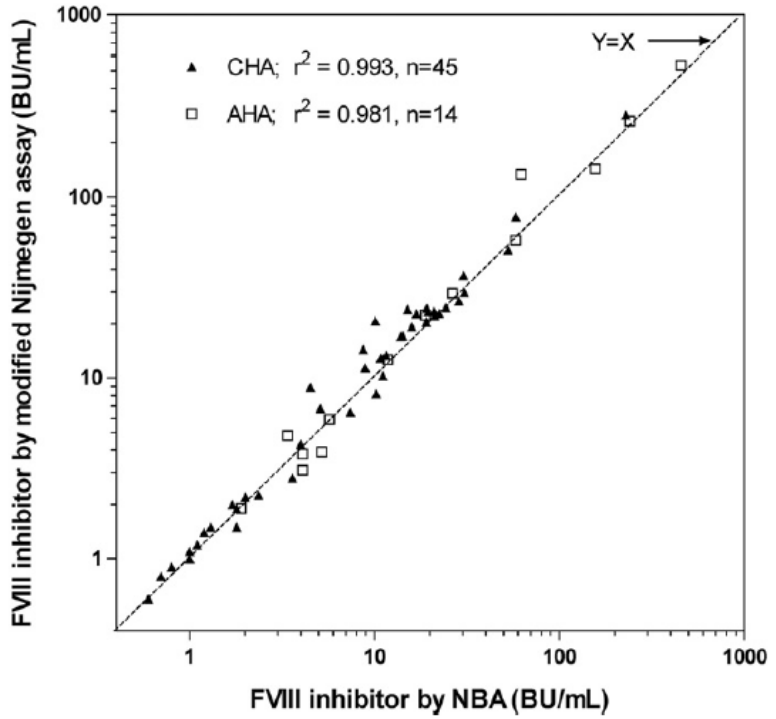
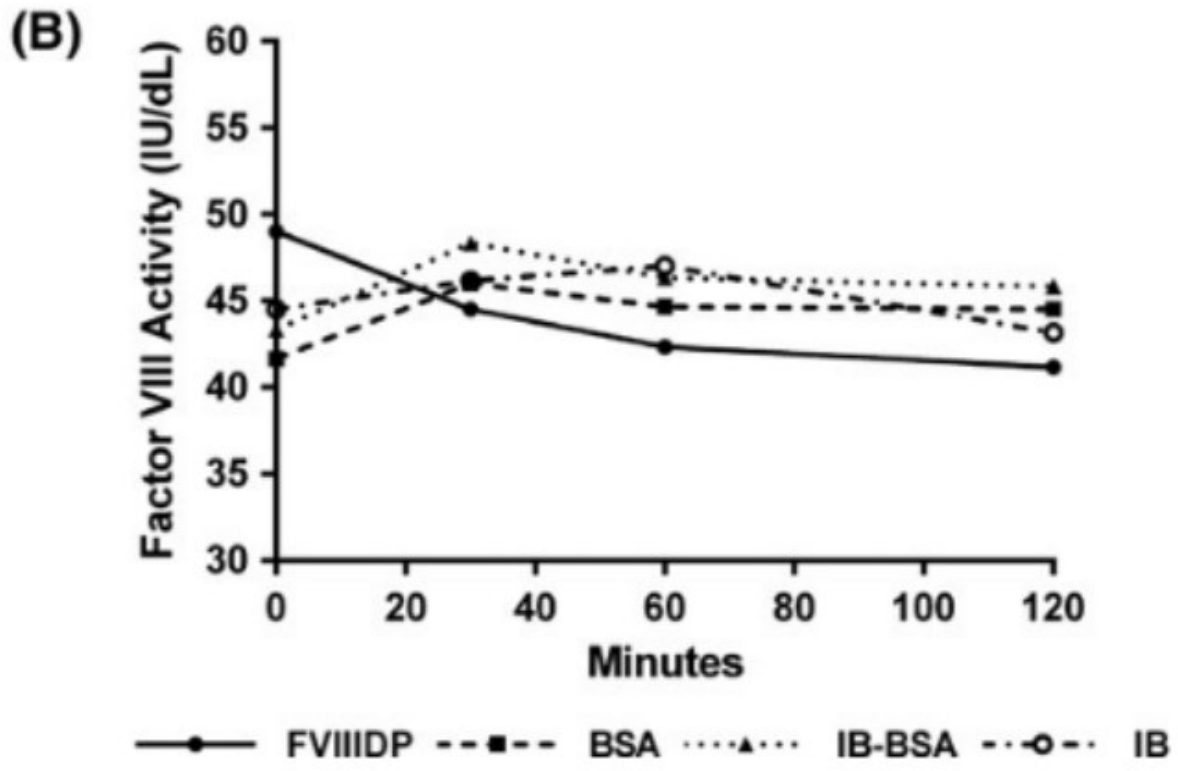


Fig. 2. Scatter plot comparing FVIII inhibitor titres obtained by the modified Nijmegen assay versus the Nijmegen Bethesda Assay.

Verbruggen, B. *et al* Thromb Haemost, 2002; 88: 362-4

Kershaw, G.W. *et al* Thromb Res, 2013; 132: 735-41

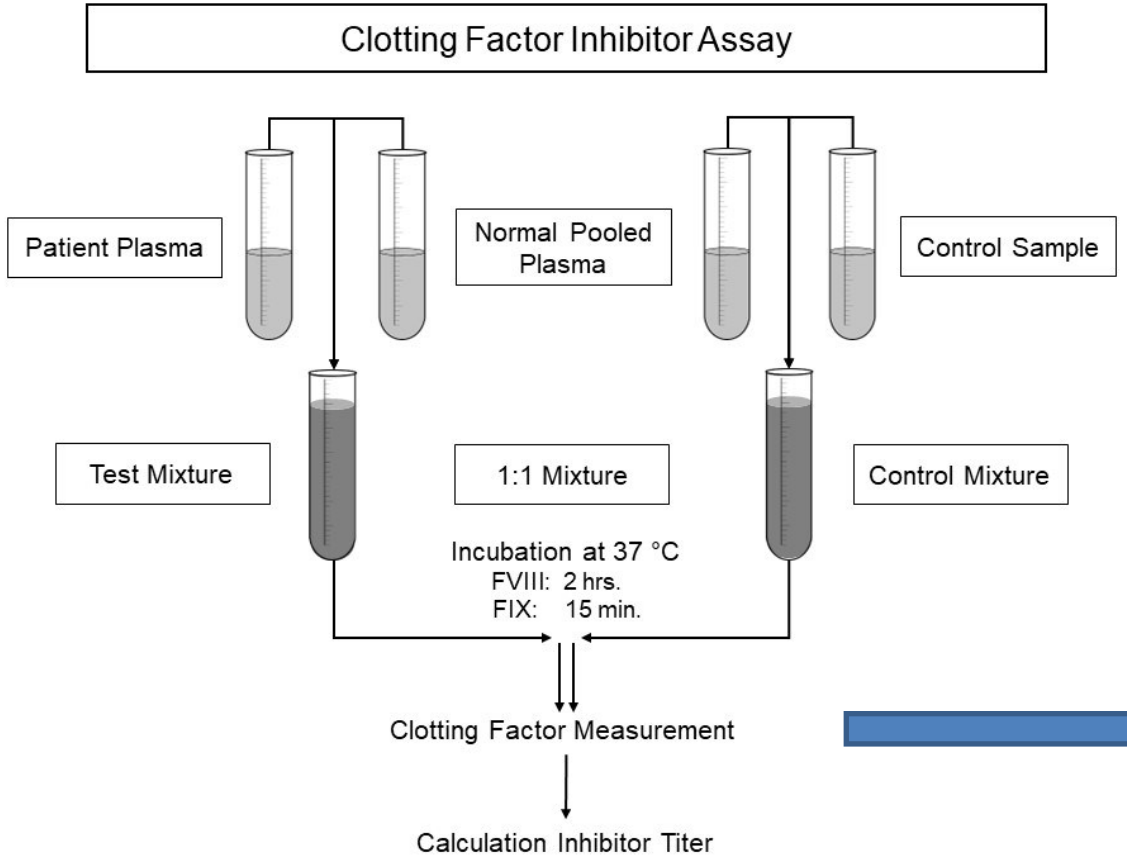
Testing requirements and interpretation: control mixture



It has been demonstrated that 4% BSA buffered with Imidazole gave more stable results than buffered FVIII-depleted plasma. Buffered 4% BSA is therefore a reliable and cost-effective substitute for FVIII-depleted plasma and supports method standardisation.

Miller, C.H. *et al* Haemophilia, 2018; 24: e116-e119

Testing requirements and interpretation: Residual FVIII / FIX measurement



Both the Bethesda and Nijmegen assays were developed by using a one-stage clotting factor assay.

Testing requirements and interpretation: Residual FVIII / FIX measurement

Inhibitor testing using a one-stage clotting assay has certain limitations ►

Miller, C.H. Haemophilia, 2018; 24: 186-197

Interference	Reference
Unfractionated heparin	Manco-Johnson, M.J. *et al* J Lab Clin Med, 2000; 136: 74-9
Lupus Anticoagulant	Blanco, A.N. *et al* Thromb Haemost, 1997; 77: 656-9
Non-specific coagulation inhibitors	Shaw, P.H. *et al* J Pediatr Hematol Oncol, 2008; 30: 135-41
Emicizumab	Nogami, K. *et al* J Thromb Haemost, 2018; 16: 1383-1390 Adamkewicz, J.I. *et al* Thromb Haemost, 2019; 119: 1084-1093 Lowe, A. *et al* Haemophilia, 2020; 26: 1087-1091

Testing requirements and interpretation: Residual FVIII / FIX measurement

It has been demonstrated that FVIII / FIX inhibitors can be reliably measured using a chromogenic assays without the risk of interferences.

Miller, C.H *et al* J Thromb Haemost, 2013; 11: 1300-9

Potgieter, J.J. *et al* Eur J Haematol, 2015; 94 Suppl 77: 38-44

Barnbrock, A., C. *et al* Haemophilia, 2016; 22: e437-9

ICSH INTERNATIONAL COUNCIL FOR STANDARDIZATION IN HAEMATOLOGY

Consensus recommendations on testing requirements and interpretation

1. A normal pooled plasma with a FVIII or FIX level of 1 ± 0.05 IU/mL should be used.
2. Imidazole-buffered (final concentration: 0.1M) normal pooled plasma with a pH between 7.3 and 7.5 should be used.
3. A control mixture of 4% bovine serum albumin buffered with imidazole should be used as a control mixture.
4. Both patient sample and control mixture are mixed in a 1:1 ratio with normal pooled plasma.
5. Both the test sample and control mixture should be incubated for 120 minutes at 37°C. For FIX an incubation of 30 min. is sufficient.

ICSH INTERNATIONAL COUNCIL FOR STANDARDIZATION IN HAEMATOLOGY

Consensus recommendations on testing requirements and interpretation

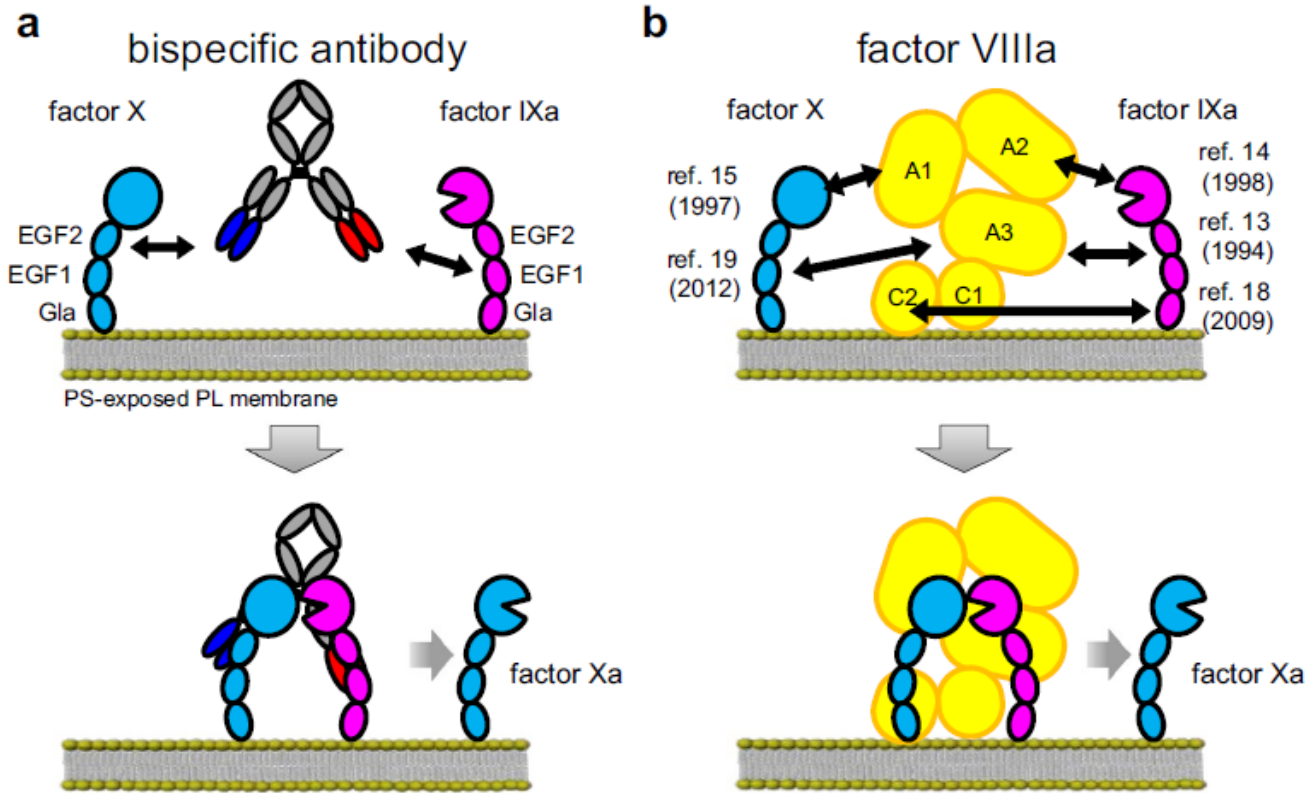
6. Either a one-stage clotting assay or a chromogenic assay can be used for the measurement of residual FVII or FIX. However, the use of a chromogenic assay may reduce the number of false-positive inhibitor results. Chromogenic assay with bovine based reagents should be used for FVIII inhibitor measurements in patients treated with emicizumab.

7. An inhibitor titre should be calculated from a sample with a %RA between 25% and 75%.

8. In the case of multiple dilutions, the sample with the least dilution close to a %RA of 50% should be used.

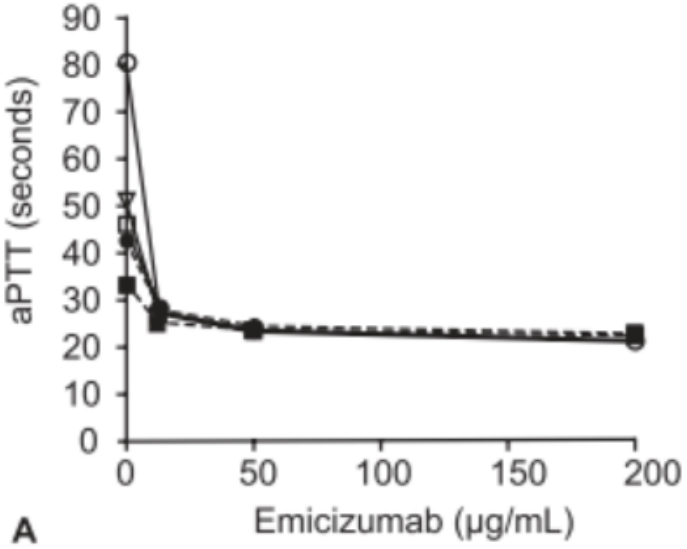
9. The threshold for positivity is 0.5 BU/ml for FVIII inhibitors and 0.3 BU/mL for FIX inhibitors.

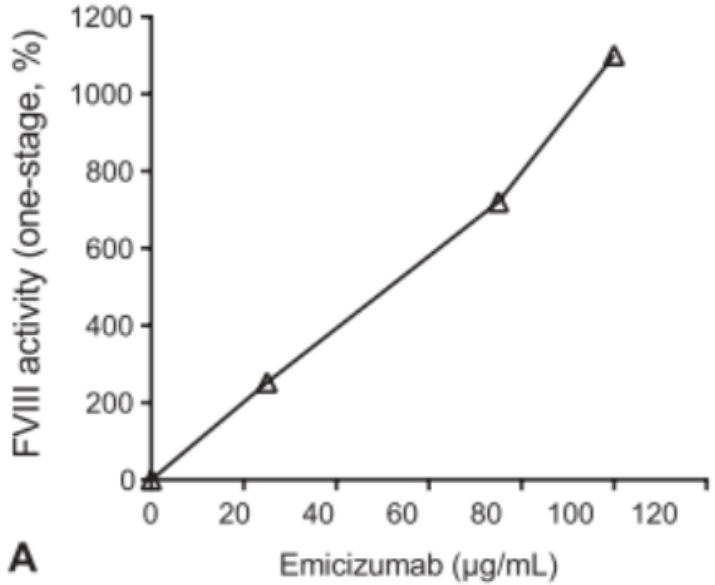
Interferences: Emicizumab

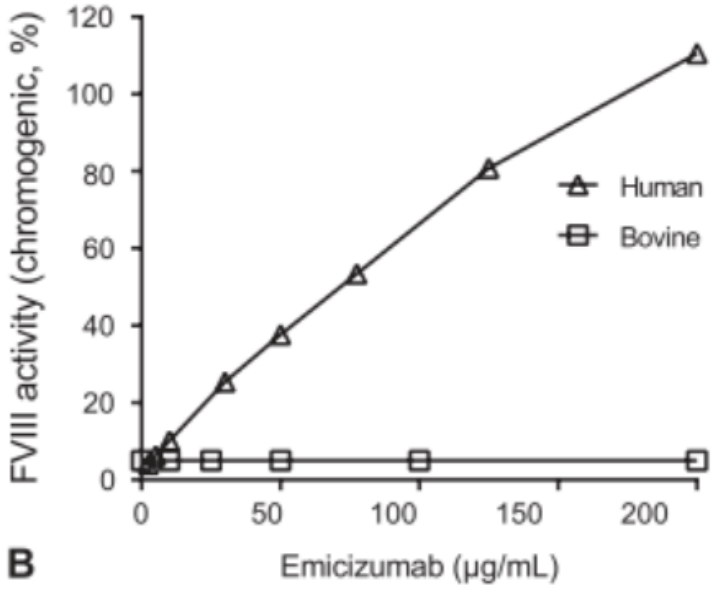


Kitazawa, T. *et al* Int J Hematol, 2020; 111: 20-30

Interferences: Emicizumab







FVIII inhibitors can only be measured reliably in the presence of emicizumab if a chromogenic method with bovine components is used.

Adamkewicz, J.I. *et al* Thromb Haemost, 2019; 119: 1084-1093

Summary of a standardised

FVIII and FIX inhibitor assay

	Factor VIII Inhibitor	Factor IX Inhibitor
Sample	Citrated plasma	Citrated plasma
Pre-heat treatment	30 minutes at 56 °C Centrifugation for 2 min. at 4000 xg	30 minutes at 56 °C Centrifugation for 2 min. at 4000 xg
Sample dilution	Unknown sample: Dilution range Known inhibitor range: Fixed dilution factor	Unknown sample: Dilution range Known inhibitor range: Fixed dilution factor
Normal pooled plasma	FVIII level between 0.95 and 1.05 U/mL	FIX level between 0.95 and 1.05 U/mL
Buffering normal pooled plasma	Imidazole buffered (final concentration: 0.1M); pH: 7.3 – 7.5	Imidazole buffered (final concentration: 0.1M); pH: 7.3 – 7.5
Control mixture	FVIII deficient plasma or 4% bovine albumin buffered with Imidazole	FIX deficient plasma or 4% bovine albumin buffered with Imidazole
Ratio for patient and control mixture	1:1	1:1
Incubation	120 min. at 37°C	30 min. at 37°C
Measurement of residual clotting factor activity	One-stage clotting assay or chromogenic assay Note: to reduce the effect of interferences the use of a chromogenic method is recommended	One-stage clotting assay or chromogenic assay Note: to reduce the effect of interferences the use of a chromogenic method is recommended
Residual factor activity	Between 25% and 75%	Between 25% and 75%
Calculation of inhibitor titre	(2-log %RA)/0.301	(2-log %RA)/0.301

	Pre-Workshop Survey (2009)		Workshop (2009)	results	Post-workshop survey (2010)	Standardized final survey 2012
Sample no. and nominal inhibitor activity	51 Laboratories	15 laboratories selected for the workshop	First Session	Last Session	13 Laboratories	22/51 Laboratories
1 1.6 BU/ml	2.3 (36%)	2.7 (43%)	3.0 (39%)	1.9 (8 %)	2.9 (41%)	2.7 (31%)
2 0.8 BU/ml	0.8 (49%)	1.0 (31%)	1.3 (69%)	0.9 (5%)	1.1 (88%)	0.7 (17%)
3 1.4 BU/ml	1.0 (41%)	1.2 (39%)	1.2 (30%)	1.2 (6%)	1.1 (31%)	1.0 (23%)
4 0.7 BU/ml	0.4 (70%)	0.6 (69%)	0.6 (45%)	0.5 (13%)	0.6 (61%)	0.5 (30%)
5 1.9 BU/ml	1.7 (36%)	1.7 (37%)	2.3 (41%)	2.2 (12%)	1.9 (31%)	1.8 (22%)
6 15.4 BU/ml	11.0 (36%)	11.5 (44%)	14.9 (41%)	14.6 (6%)	12.0 (36%)	12.4 (27%)
Mean CV	**45%**	**44%**	**44%**	**8%**	**48%**	**25%**

New development

Proof of principle of a fast and fully automated FVIII functional inhibitor test

B. VERBRUGGEN[1], N. B. BINDER [2], P. VAN VELP [2],R. POLENEWEN[1], and G. W. MOORE [2,3,4]
[1] Hemostad®, Nijmegen, The Netherlands ; [2]Technoclone Herstellung von Diagnostika und Arzneimitteln GmbH, Vienna, Austria;
[3]Addenbrooke's Hospital, Cambridge, UK; [4]Middlesex University, London, UK

Hypothesis:
"We hypothesize that testing in a VWF-free assay matrix using recombinant (r)FVIII can dramatically lower incubation time that, together with full automation, will substantially improve standardisation."

Method:
1. Predilution of sample if needed
2. Mixing with von Willebrand-free and Imidazole-buffered rFVIII (1.0 IU/mL)
3. Incubation for 5 minutes at 37°C
4. Dilution of incubated samples 1:10 with Imidazole buffer, pH 7.3. and analysis for residual rFVIII activity
   Residual FVIII activity was measured with a one-stage clotting assay.

Figure 1.
Linearity of diluted inhibitor plasma

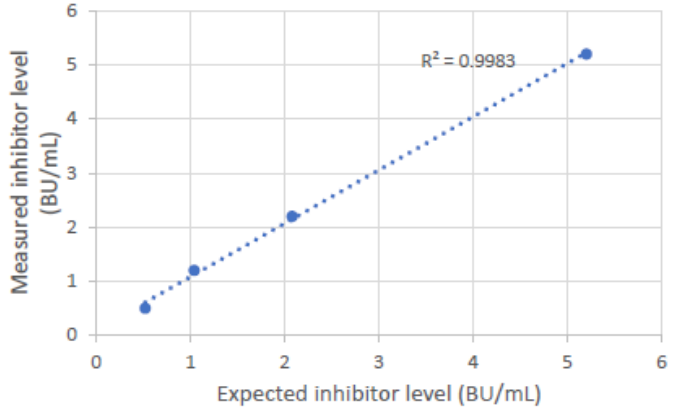
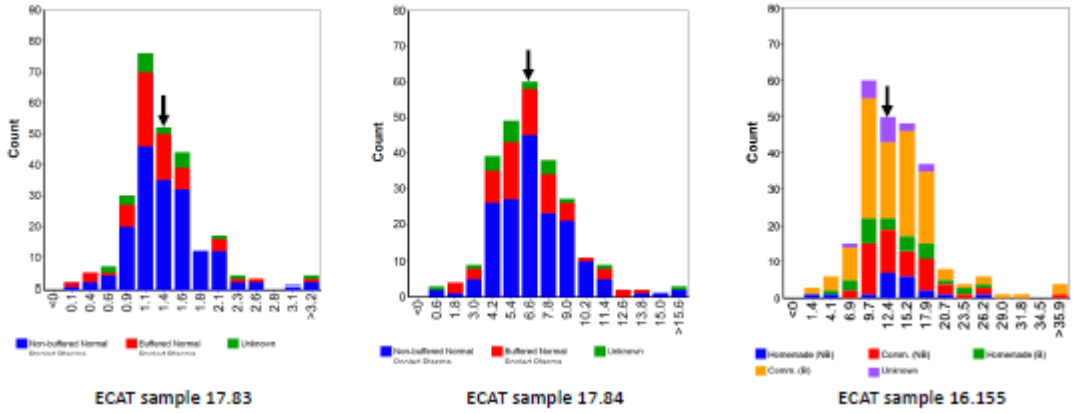


Figure 2.
ECAT reports
(with permission)



Thank you for your attention