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Collaborative Study for Value Assignment of the 4th International Standard for Factor II and X, Concentrate

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This document has been prepared for the purpose of inviting comments and suggestions on the proposals contained therein, which will then be considered by the Expert Committee on Biological Standardization (ECBS). Comments MUST be received by 01 October 2012 and should be submitted electronically to the Responsible Officer: Dr Ana Padilla at email: padillaa@who.int, with a copy to Dr David Wood at email: woodd@who.int.

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Summary

Twenty-eight laboratories from 14 different countries participated in a collaborative study to value assign the proposed 4th International Standard for Blood Coagulation Factors II and X, Concentrate (11/126, sample A) against the 3rd International Standard for Blood Coagulation Factors II and X, Concentrate (98/590). A second test sample was included in the study (11/180, sample B) to aid evaluation of assay performance.

The intra-laboratory variability was low. Over 70% of the laboratories obtained potency estimates that had intra-laboratory geometric coefficients of variation (GCV) of less than 5% when the test samples were assayed against the 3rd International Standard, indicating that the participants performed assays for factors II and X reproducibly and with high precision.

For both factors II and X, inter-laboratory variability was low for estimates of the proposed 4th International Standard against the 3rd International Standard (GCV <5%), with sample B having similarly low GCV of less than 6%.

There was a small but significant difference observed for the candidate, A (11/126), between clotting and chromogenic methods for both factors II and X. With only two laboratories performing APTT, there were insufficient data to statistically assess any differences in this case. For factor II, the overall potency from all methods was 9.44 IU/ampoule, with inter-laboratory GCV of 3%. The estimate from the Prothrombin Time based assay (9.53 IU/ampoule) was 1% higher, and the chromogenic potency (9.24 IU/ampoule) was 2% lower. For Factor X, the overall potency was 8.13 IU/ampoule, with the Prothrombin Time and chromogenic potencies being 8.29 and 7.81 IU/ampoule (2% higher and 4% lower), respectively, and inter-laboratory GCV being 5%. Taking into consideration the low inter-laboratory variation and the relatively small discrepancies between the potencies by the different methods, we propose to assign the overall geometric mean potency of 9.4 IU/ampoule for FII and 8.1 IU/ampoule for FX.

It is therefore recommended that the proposed 4th International Standard for Blood Coagulation Factors II and X, Concentrate (11/126) be assigned with potencies for functional activity, calculated relative to the 3rd International Standard for Blood Coagulation Factors II and X, Concentrate:

Factor	Proposed 4th IS IU/ampoule
II	9.4
X	8.1

Introduction

Prothrombin Complex Concentrates (PCC) are blood products composed of vitamin K dependent clotting factors II, VII, IX and X. PCC products can be used to treat factor II or X deficiencies, however by far the most common usage is for the urgent reversal of anticoagulant therapy (such as warfarin) in patients[1]. Intracranial haemorrhage is a relatively common side effect of warfarin treatment, with rates reportedly as high as 1%[1], so quick, reliable and effective reversal of warfarin treatment is essential to prevent such outcomes. Accurate measurement of clotting factor activity is essential for the safe use of these products and international standards for clotting factors, assigned in international units (IU) have been used to reduce variability in measurements both within and between laboratories. Monocomponent Factor IX concentrates are used as replacement therapy for Haemophilia B patients. These concentrates contain only factor IX and the manufacturers are required to control the levels of factors II, VII and X to ensure the

safety of these products. The current international standard for Factors II and X, Concentrate is used mainly by therapeutics manufacturers and regulators to ensure the continuity of factor II and X activity measurements within such products and enable appropriate dosage to patients.

The 3rd International Standard (IS) for Factors II and X, Concentrate (98/590) was established by the Expert Committee on Biological Standardisation (ECBS) of the World Health Organisation (WHO) in October 1999. Due to dwindling stock, it is now necessary to replace this IS and this study served to value assign a replacement IS for FII and FX against the 3rd IS. Another Factors II and X concentrate, sample B (11/180) was included as a test sample for information only to further evaluate the performance of the assay methods and the candidate IS.

Participants

Twenty-eight laboratories from 14 different countries (3 Austria, 1 Canada, 2 China, 1 Croatia, 4 France, 4 Germany, 1 Italy, 1 Japan, 1 Korea, 1 The Netherlands, 2 Spain, 1 Sweden, 3 UK, 3 USA) agreed to participate in the study, all of which returned data in time for the statistical analysis. One laboratory (Lab 28) carried out single point estimations and was therefore excluded from the analysis.

The participants included 4 clinical laboratories, 7 diagnostics manufacturers, 10 therapeutic manufacturers and 7 regulatory authorities. A list of participants is given in Appendix 1 at the end of this report. Each laboratory is referred to in this report by an arbitrarily assigned number, not necessarily representing the order of listing in the Appendix.

The Candidate, NIBSC code 11/126

Materials were donated by Grifols Biologicals Inc., BioProducts Laboratory and Baxter AG. Due to the availability of bulk material, one candidate progressed to definitive fill. The single batch of material was diluted in 40 mM Tris, 120 mM NaCl, 1.6 mg/ml trehalose and 4 mg/ml human serum albumin. The material was distributed in glass ampoules, filled and freeze-dried according to guidelines for production of international standards[2]. All material had been tested and was found negative for anti-HIV1/2, HBsAg and hepatitis C. The candidate was coded A in the study and the product characteristics are listed in the following table.

NIBSC Code	11/126		
Presentation	Sealed, glass 5 ml DIN ampoules		
Filling date	5th May 2011		
Number of Ampoules available	15,000		
Liquid filling weight (g) (n=587, measurements taken from all 3 pumps throughout the duration of the fill)	1.0074 (Range 1.0020 - 1.0105)		
CV of fill mass (%)	0.1384		
Homogeneity of the fill by activity: 3 ampoules selected from the beginning of the fill, after every 5000 ampoules and at the end of the fill were assayed against the 3rd IS using clot-based assays. 2 assays per ampoule were carried out. Effect of fill position assessed by ANOVA of log potencies.		GCV	p
	FII	3.879	1.01
	FX	3.072	1.61
Mean dry weight (g) (n=6)	0.0255 (CV 0.84%)		
Mean head space oxygen (%) (n=12)	0.32 (CV 32.44%)		
Residual moisture (%) (n=12)	0.689 (CV 24.23%)		
Storage temperature	-20°C		
Address of processing facility	NIBSC, Potters Bar, EN6 3QG, UK		
Address of present custodian	NIBSC, Potters Bar, EN6 3QG, UK		

Samples

The following coded samples were sent to each participant:

S	3rd International Standard for Factors II & X, Concentrate (98/590), containing 11.2 IU FII and 10.2 IU FX per ampoule
A	Proposed 4th International Standard for Factors II & X, Concentrate (11/126) containing approximately 9 IU per ampoule FII and 8 IU per ampoule FX.
B	Factors II & X, Concentrate (11/180) containing approximately 11 IU FII and 7 IU FX per ampoule.
P	4th International Standard Factors II, VII, IX & X, Plasma, containing 0.89 IU per ampoule of factors II and X.

Assay Methods

Each participant was requested to perform their routine in-house functional method(s) for the two coagulation factors. Some laboratories performed more than one method and the data from each method were treated as separate sets of results and as an example referred to as Lab 19a and Lab19b. A list of reagents, methods and instruments used by the participants is given in Appendix 2.

Factor II: For samples A and B, 22 laboratories used a Prothrombin Time based clotting method with commercial thromboplastin reagents (sources were rabbit brain, human placenta or recombinant human) and FII-deficient plasma. Eleven laboratories used chromogenic assays; 10 laboratories used Ecarin-based assays, while one laboratory also employed a prothrombinase-based method (Lab 19b). Two laboratories used an Activated Partial Thomboplastin Time method (APTT) using commercial reagents. For sample P, 22 laboratories carried out the Prothrombin Time based assays, while 10 laboratories employed Ecarin chromogenic assays.

Factor X: For samples A and B, a one-stage clotting assay based on Prothrombin Time was used by 22 laboratories, using a variety of thromboplastin reagents (rabbit brain, human placenta or recombinant human), and FX-deficient plasma. Ten laboratories used chromogenic methods, using Russell's Viper Venom as an activator and two laboratories used APTT using commercial reagents. For sample P, 22 laboratories carried out the Prothrombin Time based assays, while 9 laboratories employed Ecarin chromogenic assays

Study Design

Participants were requested to carry out four assays for each factor (FII & FX) using fresh ampoules of samples S, A, B, and P in each assay. Laboratories were requested to assay factors II and X on the same ampoules. Within each assay, participants were requested to assay three dilutions of each of the samples S, A, B and P, in replicate, according to balanced assay designs (study protocol shown in Appendix 4).

Raw assay data were returned together with calculated estimates for samples A (proposed 4th IS, 11/126), B (11/180), and P (09/172) relative to sample S (3rd IS, 98/590) from each individual assay.

Statistical Analysis

An independent statistical analysis of raw data was performed at NIBSC. Potency estimates relative to the 3rd International Standard (98/590), were calculated by parallel-line analysis[3] of log transformed assay response (clotting time or absorbance) against log concentration, independently for each test sample included in each assay. Assay validity was assessed by analysis of variance and any deviations from linearity and parallelism were considered significant at the 1% level ($p<0.01$). Where significant deviations from the model appeared to result from underestimation of residual error, linearity was assessed by visual inspection of the plotted data and non-parallelism was assessed using deviations from linearity as an alternative residual error. Any assays rejected for deviations from linearity or parallelism are indicated in the tables of results. Results from all valid assays were combined to generate unweighted geometric mean potencies for each laboratory and these laboratory means were used to calculate overall unweighted geometric mean potencies. Variability between assays and laboratories has been expressed using geometric coefficients of variation ($GCV = \{10^s-1\}\times100\%$ where s is the standard deviation of the $\log_{10}$ transformed potency potencies). Grubbs' Test[4] was applied to the log transformed laboratory mean potencies in order to detect any significant outliers. Comparisons between methods have been made by unpaired t-tests of log transformed laboratory mean potencies. In general, the laboratories' reported potency estimates were close to those calculated centrally by NIBSC (data not shown).

Results

Assay Data

Twenty seven participants returned a total of 140 assays which comprised 72 FII assays, and 68 FX assays. Lab 28 performed single point assays and were therefore not included in the potency estimation. The individual assay results, together with the geometric mean potencies (GM) and the intra-laboratory variation expressed as %GCV are presented in Appendix 3.

Assay Validity

Except for the following, all assays were valid as defined by criteria set out in the above Statistical Analysis section of this report, .

- FII, Lab 15, Chromogenic assay, sample B, assay 1, non-parallelism
- FII, Lab 21, Prothrombin Time based assay, sample B, assay 4, non-linearity
- FX, Lab 3, Chromogenic assay, sample A, assay 4, non-linearity
- FX, Lab 3, Chromogenic assay, sample B, assay 4, non-linearity
- FX, Lab 3, Chromogenic assay, sample P, assay 4, non-linearity
- FX, Lab 15, Chromogenic assay, sample P, assay 2, non-linearity

The proposed 4th IS for Factors II and X, Concentrate (sample A, 11/126)

Intra- and inter- laboratory variability

Estimates of intra-laboratory variability (between assays) for both factors II and X in sample A are given as geometric coefficients of variation (GCV) for potency estimates relative to sample S (Table 1a). These exceeded 5% in 7 (8 including the outlier) and 7 laboratories for FII and FX respectively, with 80% of cases giving GCV lower than 5%. As shown by the range of intra-laboratory GCVs, the within laboratory reproducibility for the Prothrombin Time based assays,

chromogenic methods and APTT were similar and there were no obvious differences between the reproducibility of the factor II and factor X assays.
Inter-laboratory variability was low for both factors II and X, being 3.1 and 4.6%, respectively, showing good agreement between laboratories.

Potency estimates

The overall potency estimates for sample A (proposed 4th IS) were calculated relative to the assigned values for the 3rd IS. Laboratory mean potency estimates and overall potency estimates, together with 95% confidence limits, for factors II and X are shown in Table 1a. The potency estimates relative to the 3rd IS from individual assays are also presented in histogram form in Figures 1a – 1b. The histograms illustrate good agreement between laboratories for sample A relative to the 3rd IS for both factors; with only one outlier detected by Grubbs' test (Lab 12) for each factor.

Factor II

Outlier detection indicated that the FII estimate of potency by Lab 12 (Prothrombin time based clotting assay) was significantly lower than all other FII estimates. These results were therefore excluded from the calculations of overall mean potency estimates of 9.44 IU/ampoule for FII.

Comparison of the potency estimates by Prothrombin Time assays with results from the chromogenic assays showed that there was a statistically significant difference in the potencies (Table 2a). The geometric mean potency by clotting assay was 9.53 IU/ampoule compared to 9.24 IU/ampoule for chromogenic assays (p=0.004). As only two laboratories carried out the APTT clotting assay, there was not enough data to assess significance in this case. The overall geometric mean for all methods was 9.44 IU/ampoule. The difference between the overall geometric mean and the Prothrombin Time geometric mean potency was 0.95%, and for the chromogenic potency, 2.1%.

Factor X

Lab 12 was found to have significantly lower results than the other estimates, and was therefore excluded from the overall potency calculation.

Comparison of the Prothrombin Time and chromogenic assays showed that there was a significant difference between the potencies from the two methods (Table 2a; p<0.001). As with factor II, APTT methods were carried out by only two laboratories and therefore the differences in potency estimates were not assessed. The overall geometric mean for all methods was 8.13 IU/ampoule. The difference between the overall geometric mean and the Prothrombin Time geometric mean potency was 2.0%, and for the chromogenic potency 4.0%

Sample B, Factors II and X, Concentrate (11/180)

Intra- and inter- laboratory variability

Estimates of intra-laboratory variability for both factors II and X in sample B are given as geometric coefficients of variation (GCV) for potency estimates relative to sample S (Table 1b). These exceeded 5% in only 7 and 5 laboratories for FII and FX respectively, with 80% of cases giving a GCV lower than 5%.

Inter-laboratory variability was low at 2.5 and 4.6%, respectively, for factors II and X, showing good agreement between laboratories.

Potency estimates

The overall potency estimates and 95% confidence limits for test sample B calculated relative to the assigned values for the 3rd IS are shown in Table 1b, together with laboratory mean potency estimates. The potency estimates relative to the 3rd IS from individual assays are also presented in histogram form in Figures 1c – 1d. The histograms illustrate good agreement between laboratories for sample B relative to the 3rd IS for both factors; with only one outlier (Lab 12) for factor X only.

Factor II

The overall mean potency by all methods was estimated to be 11.08 IU/ampoule. No outliers were detected for factor II assays. Comparison of the Prothrombin Time based assay laboratory mean potencies with those obtained using the chromogenic assay revealed no significant difference between estimates (Table 3a; p=0.793).

Factor X

The mean potency estimate from Lab 12 was found to be significantly lower than the estimates from the other laboratories, and was therefore excluded from the overall potency calculation. The difference between the Prothrombin Time based and chromogenic assay mean potencies was significant at the 5% level (Table 3a; p=0.037). The overall mean potency by all methods was calculated to be 6.92 IU/ampoule.

Sample P, 4th IS Factors II, VII, IX and X, Plasma (09/172)

Intra- and inter- laboratory variability

Estimates of intra-laboratory variability for both factors II and X in sample P are given as geometric coefficients of variation (GCV) for potency estimates relative to sample S (Table 1c). These exceeded 5% in 13 and 10 laboratories for FII and FX respectively, with over 60% of cases giving a GCV lower than 5%.

Inter-laboratory variability, at 8.3 and 7.4%, respectively, for factors II and X was higher than that obtained for concentrate samples (samples A and B).

Stability study

An accelerated degradation study of 11/126 was carried out at NIBSC using clot based assays (Prothrombin time) for FII and FX. Data from 2 time-points (6 months and 12 months) indicate that the material is stable, with the following table showing predicted loss per year relative to samples stored at -150°C. Two assays were carried out on each ampoule for each factor, for each temperature.

Temperature	**F II**		**FX**	
	Predicted %Potency Loss per year	**95% Upper confidence limit of Potency Loss**	**Predicted %Potency Loss per year**	**95% Upper confidence limit of Potency Loss**
-20°C	0.005	0.016	0.006	0.019
4°C	0.231	0.537	0.254	0.599
20°C	2.056	3.769	2.159	4.025

The potencies per ampoule, relative to the -150°C sample, are shown in the following table (storage for 12 months at accelerated temperatures):

Storage temperature	FII potency (relative to -150°C)	Confidence limits (95 %)	FX potency (relative to -150°C)	Confidence limits (95 %)
-70°C	1.06	1.00-1.12	1.04	0.98-1.09
-20°C	1.04	0.99-1.10	1.02	0.97-1.07
4°C	1.00	0.91-1.11	1.03	0.98-1.09
20°C	0.95	0.90-1.00	0.99	0.94-1.04
37°C	0.87	0.82-0.92	0.87	0.83-0.92
45°C	0.65	0.62-0.69	0.66	0.63-0.70
56°C	0.30	0.25-0.29	0.28	0.26-0.29

No loss of activity is observed after storage up to 20°C after 1 year (confidence limits all overlap 1.0). Continual real time degradation studies of the –20°C against ampoules stored at –150°C and further accelerated degradation studies at elevated temperatures will be carried out to monitor the stability of the replacement standard.

Assessment of on-bench stability was carried out at NIBSC by storage of the ampoule on melting ice, and the activity of FII and FX monitored at 0 h, 1 h, 2 h and 3 h by assessment of activity relative to a fresh ampoule of 11/126. Two assays were carried out for each factor, and the percentage potency estimates at each time-point are shown below:

Time	FII (% potency)	Confidence limits (95 %)	FX (% potency)	Confidence limits (95 %)
0 h	109.0	100.2-121.4	98.9	91.7-106.6
1 h	99.7	94.7-105.6	97.9	93.2-103.0
2 h	95.5	90.0-101.3	101.6	97.6-105.7
3 h	99.4	93.0-106.1	100.8	96.2-105.6

Other than a slightly high measurement of FII potency at 0 h, (where the confidence limits are from 100.2 to 121.4%), all potency estimates were close to 100% and all confidence limits overlapped 100% at all time-points. This shows that the material is stable up to 3 hours storage on melting ice.

Discussion

The primary aim of this study was to value assign a replacement International Standard for Factors II and X, Concentrate. Another concentrate test sample was included in the study to provide further information on the variability of assay and relationship between unit assignment.

For both factors II and X, over 60% of the participants carried out Prothrombin Time based clotting assays, while 30% of the laboratories carried out chromogenic assays. In contrast to the previous study that established the 3rd IS, there was an increase in the proportion of laboratories that employed the chromogenic assays[5]. This may be a reflection of the establishment of the chromogenic assays as European Pharmacopoeial monograph methods for factors II and X. The within laboratory reproducibility of all the methods for all the samples against the 3rd IS was excellent, with the majority of the laboratories obtaining GCVs of less than 5%. Interestingly, higher variability was expected for sample P, the 4th IS for Factors II, VII, IX and X, Plasma,

when it was assayed against the concentrate IS, however, the intra-laboratory GCVs for sample P, were generally as low as those obtained for the concentrate samples.

Agreement between laboratories was excellent when sample A, the proposed 4th IS, was assayed against the 3rd IS; the inter-laboratory variability, expressed as GCVs, was 3.1% and 4.6% for FII and X respectively (Table 1a). Similarly good agreement was obtained for sample B, with 2.5% GCV for FII and 4.6% for FX (Table 1b). As expected, higher inter-laboratory variability for estimates of the plasma IS, sample P, than for the concentrate samples (A and B) were obtained, with GCVs of 8.3% and 7.4% for FII and X respectively (Table 1c). This reinforces the importance of assaying "like against like" for good laboratory agreement of potency estimates.

For samples B and P, there was no significant discrepancy between potencies obtained by the different methods for both FII and X, at the 1% level (Tables 3 and 4). However, for sample A, the proposed 4th IS, there was a significant difference between the potencies estimated by the Prothrombin Time based assays and the chromogenic assays (Table 2a). For FII, there was a 3% difference in the values obtained by these two different types of methods, the ratio of clotting to chromogenic potency being 1.031. The large number of laboratory results and the extremely low level of variability between them (inter-laboratory GCV of less than 3% for both Prothrombin Time based and chromogenic assays) is a major contributing factor to the statistical significance of this discrepancy. Compared with the overall mean potency of 9.44 IU/ampoule, calculated using estimates from all methods, the differences in estimates to the Prothrombin Time based assays, chromogenic assays and APTT were +0.95%, -2.12% and +1.80% respectively (Table 2b). Taking into account the inter-laboratory variation of 3.1% for all methods and the % discrepancies by the different methods relative to the overall mean potency, the 3% difference in potency estimates by the Prothrombin Time based assay and chromogenic assay would not have no detectable impact on the agreement of potency estimates if the proposed 4th IS, sample A, were to be labelled with the mean value (9.44 IU/ampoule) derived from all laboratory mean potencies. An identical mean potency estimate (11.08 IU/ampoule) is obtained for sample B calculated relative to the 3rd IS or against sample A with the assumed value of 9.44 IU/ampoule (Table 2c) and the FII value assignment of the proposed 4th IS with the unweighted geometric mean estimates from all laboratories mean will ensure the continuity of the FII concentrate unit. For FX, the difference in values obtained by the Prothrombin Time based and chromogenic assays was 6% with the clotting to chromogenic potency ratio of 1.061 (Table 2a). Similar to the FII, the FX assay discrepancy could be due to the large number of labs with high precision assays. Compared with the overall mean potency of 8.13 IU/ampoule, calculated using estimates from all methods, the differences in estimates to the Prothrombin Time based assays, chromogenic assays and APTT were +1.97%, -4.10% and +1.11% respectively (Table 2d). These differences are well within the inter-laboratory variability of 4.6% and would not have practical effect on the agreement of potency if the proposed 4th IS, sample A were to be labelled with the mean value (8.13 IU/ampoule) derived from all laboratory mean potencies. This is evidenced by the mean potency estimates of 6.92 and 6.91 IU/ampoule obtained for sample B when assayed against the 3rd IS and against sample A with the assumed value of 8.13 IU/ampoule, respectively (Table 2e).

For the 4th IS for FII, VII, IX and X, Plasma, sample P, the estimated FX potency in this study is the same as its assigned value of 0.89 IU/ampoule. However, for FII, the estimated potency was 0.82 IU/ampoule and this is 8% lower than its assigned value of 0.89 IU/ampoule. Unpaired t-test of log potencies obtained from the current study and the study that established the 4th IS for FII, VII, IX and X, Plasma[6] showed that this difference is statistically significant ($p<0.001$). A similar discrepancy was observed in the study that established the 3rd IS for FII and X Concentrate in 1999. In that study, the 2nd IS for Factors II, VII, IX and X, plasma, 94/764, was assayed against the 2nd IS for Factors II and X, concentrate, 84/683 and the estimated potencies for

FII and FX were 10% and 4% respectively lower than its assigned potencies. The only previous occasion that concentrate and plasma standards have been compared was when the 1st IS for both plasma and concentrate were established in 1986; at that time there was complete agreement between the two types of standard. However, since then both the production methods and assay methods have changed, and it is possible that these changes may have contributed to the discrepancies seen in the 1999 and the current studies. A second possibility is that the plasma standard established in 2010 may have lost some activity. This is unlikely as accelerated degradation study of the 4th IS for FII, VII, IX and X, Plasma showed that this standard would be very stable, with a predicted loss of FII activity of 0.09% per year at the storage temperature of -20°C. So it is more likely that the divergence of the plasma and concentrate unit of FII was due to changes in assay methods and happened as far back as 1999. It is encouraging that the differences in both the FII and FX plasma and concentrate IU found in the 1999 study have shown to be reduced in the current study from 10 to 8% for FII and from 4%to 0% for FX.

The ratio of potency estimates from the clotting and chromogenic methods can give an indication of the activation status of a preparation of purified coagulation factor and typically this calculation has been used to assess the activation status of factor VII[7]. For example, a clotting/chromogenic ratio >1.0 indicates that FVII in the test sample is relatively more activated than the reference standard. In the current study, the clotting and chromogenic assay potency ratios for all samples were close to 1 (Table 2a, 3a and 4a) suggesting the absence of activated FII and X in these preparations.

Preliminary accelerated degradation study data indicated that sample A, 11/126 is stable with less than 0.01% predicted loss of activity per year when the ampoules are stored at -20° and as with all international standard, the stability of this material will be assured by real-time monitoring.

Proposal to Participants

Although there were statistically significant differences in the potencies obtained using the Prothrombin based clotting assays and Chromogenic methods for both FII and X for the candidate sample, the discrepancies were relatively small with no practical significance and result from the low level of inter-laboratory variability. It was therefore recommended that a single mean potency, calculated using all laboratory mean estimates be assigned to each analyte. The potencies were assigned relative to the 3rd International Standard for Factors II and X, Concentrate, thus the integrity of the International Unit has been maintained. The proposed overall mean potencies for functional activity for the two factors were: Factor II – 9.4 IU/ampoule and FX – 8.1 IU/ampoule.

Responses from participants and the experts nominated by the SSC/ISTH Plasma Coagulation Inhibitors Sub-Committee

All participants and SSC nominated experts who sent in responses agreed with the proposals. There were no comments in relation to the analysis or interpretation of the data in the study.

Proposal and Recommendation to the ECBS

Sample A, 11/126, be the 4th International Standard for Factors II and X, Concentrate with the following potencies assigned relative to the 3rd International Standard for Factors II and X, Concentrate (98/590):

Factor II: 9.4 IU/ampoule
Factor X: 8.1 IU/ampoule

The Instruction for Use for the proposed Standard, 11/126 is illustrated in Appendix 5.

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Table 1a: Potency estimates, intra- and inter-laboratory GCV for factors II and X in sample A relative to sample S, the 3rd IS for Factors II and X, Concentrate

Assay Method	Lab	FII		FX	
		IU/amp	GCV	IU/amp	GCV
PT	01	9.52 (n=4)	2.7%	8.13 (n=4)	6.3%
	04	9.40 (n=4)	2.1%	8.43 (n=4)	2.5%
	05a	9.69 (n=4)	3.5%	8.34 (n=4)	3.4%
	06	9.54 (n=4)	0.8%	8.18 (n=4)	0.7%
	07b	9.34 (n=4)	10.1%	9.09 (n=4)	6.1%
	08	9.10 (n=4)	4.4%	8.02 (n=4)	0.9%
	11	9.75 (n=4)	5.4%	8.22 (n=4)	5.3%
	12	7.66 (n=4)	5.3%	6.81 (n=4)	1.0%
	13	9.72 (n=4)	4.5%	8.44 (n=4)	1.5%
	14a	9.56 (n=4)	2.0%	8.65 (n=4)	2.7%
	14b	9.97 (n=4)	3.0%	8.47 (n=4)	3.5%
	16a	9.50 (n=4)	3.7%	8.27 (n=4)	2.2%
	16b	9.44 (n=4)	4.1%	8.02 (n=4)	3.5%
	16c	9.39 (n=4)	7.0%	8.27 (n=4)	2.5%
	16d	9.08 (n=4)	7.4%	8.10 (n=4)	3.2%
	17	10.08 (n=4)	2.0%	7.88 (n=4)	1.9%
	18	9.64 (n=4)	2.5%	8.46 (n=4)	1.6%
	20	9.04 (n=4)	3.4%	8.03 (n=4)	2.0%
	21	9.47 (n=4)	1.3%	8.40 (n=4)	6.7%
	24	9.45 (n=4)	5.2%	8.39 (n=4)	5.8%
	25a	9.86 (n=4)	3.5%	8.34 (n=4)	4.4%
	26	9.74 (n=4)	1.3%	8.08 (n=4)	3.7%
CH	2	9.23 (n=4)	1.6%	7.86 (n=3)	2.3%
	3	9.13 (n=4)	5.5%	7.67(n=3)**	3.3%
	5b	8.92 (n=4)	1.8%	7.50 (n=4)	3.1%
	9	8.99 (n=4)	2.0%	8.08 (n=4)	0.7%
	10	9.24 (n=4)	0.7%	7.80 (n=4)	6.0%
	15	9.24 (n=3)*	2.9%	7.73 (n=4)	1.7%
	19a	9.25 (n=4)	1.9%	8.33 (n=4)	1.7%
	19b	9.67 (n=4)	2.3%	NT	
	22	9.31 (n=4)	1.5%	7.21 (n=4)	1.6%
	23	9.29 (n=4)	7.7%	7.71 (n=4)	4.8%
	25b	9.42 (n=4)	1.1%	8.33 (n=4)	3.2%
APTT	7a	9.35 (n=4)	4.1%	7.81 (n=4)	0.9%
	27	9.88 (n=4)	4.5%	8.28 (n=4)	5.6%
Overall GM		9.44 (n=34)		8.13 (n=33)	
95% CL		9.34 – 9.54		8.00 – 8.26	
Between Lab GCV		3.1%		4.6%	

NT: not tested; PT: prothrombin time; CH: chromogenic assay; APTT: activated partial thromboplastin time; N/A: not applicable; GM: geometric mean; GCV: geometric coefficient of variation; CL: confidence limits. *One assay from 4 performed was not parallel to the standard sample and has been excluded from the analysis. **One assay from the 4 performed was not linear and was excluded from the analysis.
Shaded boxes indicate outliers and are excluded from overall GM.

Table 1b: Potency estimates, intra- and inter-laboratory GCV for factors II and X in sample B relative to sample S, the 3rd IS for Factors II and X, Concentrate

Assay Method	Lab	FII		FX	
		IU/amp	GCV	IU/amp	GCV
PT	1	11.03 (n=4)	5.6%	6.74 (n=4)	2.4%
	4	11.04 (n=4)	3.2%	7.21 (n=4)	1.8%
	5a	11.19 (n=4)	5.7%	7.10 (n=4)	2.1%
	6	11.25 (n=4)	1.2%	6.88 (n=4)	1.4%
	7b	11.22 (n=4)	9.5%	7.60 (n=4)	4.1%
	8	10.62 (n=4)	2.9%	6.82 (n=4)	3.0%
	11	10.82 (n=4)	5.1%	6.55 (n=4)	5.3%
	12	10.87 (n=4)	6.2%	5.03 (n=4)	2.9%
	13	11.42 (n=4)	2.8%	7.08 (n=4)	1.5%
	14a	11.40 (n=4)	3.0%	7.37 (n=4)	2.0%
	14b	11.66 (n=4)	2.6%	7.34 (n=4)	3.3%
	16a	11.31 (n=4)	3.1%	7.07 (n=4)	4.4%
	16b	10.83 (n=4)	3.1%	6.80 (n=4)	5.3%
	16c	11.04 (n=4)	4.9%	7.19 (n=4)	4.4%
	16d	10.39 (n=4)	6.5%	6.70 (n=4)	4.0%
	17	10.95 (n=4)	2.6%	6.96 (n=4)	7.9%
	18	11.18 (n=4)	4.8%	7.02 (n=4)	1.6%
	20	10.55 (n=4)	3.1%	6.70 (n=4)	2.2%
	21	10.68 (n=3)**	1.7%	7.29 (n=4)	2.4%
	24	11.12 (n=4)	6.2%	6.92 (n=4)	3.4%
	25a	11.28 (n=4)	1.3%	6.98 (n=4)	3.7%
	26	11.50 (n=4)	2.1%	6.83 (n=4)	2.8%
CH	2	11.22 (n=4)	3.2%	6.71 (n=4)	2.5%
	3	10.80 (n=4)	4.0%	6.77 (n=3)**	1.9%
	5b	10.89 (n=4)	4.4%	6.12 (n=3)	2.1%
	9	10.93 (n=4)	0.4%	6.94 (n=4)	0.8%
	10	11.14 (n=4)	1.0%	6.97 (n=4)	1.1%
	15	11.18 (n=3)*	2.6%	6.48 (n=4)	3.3%
	19a	11.14 (n=4)	2.8%	7.17 (n=4)	2.7%
	19b	11.27 (n=4)	1.3%	NT	-
	22	11.03 (n=4)	1.4%	6.33 (n=4)	2.6%
	23	11.27 (n=4)	3.1%	7.16 (n=4)	6.4%
	25b	11.09 (n=4)	4.6%	7.00 (n=4)	3.4%
APTT	7a	11.17 (n=4)	1.2%	6.56 (n=4)	1.2%
	27	11.44 (n=4)	3.7%	7.14 (n=4)	7.4%
Overall GM		11.08 (n=35)		6.92 (n=33)	
95% CL		10.99 – 11.18		6.81 – 7.03	
Between Lab GCV		2.5%		4.6%	

NT: not tested; PT: prothrombin time; CH: chromogenic assay; APTT: activated partial thromboplastin time; N/A: not applicable; GM: geometric mean; GCV: geometric coefficient of variation; CL: confidence limits. *One assay from 4 performed was not parallel to the standard sample and has been excluded from the analysis. **One assay from the 4 performed was not linear and was excluded from the analysis.
Shaded boxes indicate outliers and are excluded from overall GM.

Table 1c: Potency estimates, intra- and inter-laboratory GCV for factors II and X in sample P relative to sample S, the 3rd IS for Factors II and X, Concentrate

Assay Method	Lab	FII		FX	
		IU/amp	GCV	IU/amp	GCV
PT	1	0.79 (n=4)	7.7%	0.79 (n=4)	2.4%
	4	0.77 (n=4)	1.3%	0.86 (n=4)	1.7%
	5a	0.82 (n=4)	5.8%	0.89 (n=4)	1.7%
	6	0.81 (n=4)	0.8%	0.91 (n=4)	0.8%
	7b	0.77 (n=4)	8.1%	0.87 (n=4)	5.6%
	8	0.93 (n=4)	2.6%	0.98 (n=4)	0.4%
	11	0.78 (n=4)	6.7%	0.85 (n=4)	7.4%
	12	0.71 (n=4)	5.1%	0.91 (n=4)	7.7%
	13	0.83 (n=4)	5.2%	0.94 (n=4)	1.1%
	14a	0.92 (n=4)	3.7%	0.98 (n=4)	3.1%
	14b	1.00 (n=4)	5.1%	1.00 (n=4)	3.8%
	16a	0.76 (n=4)	1.9%	0.82 (n=4)	3.5%
	16b	0.79 (n=4)	5.6%	0.88 (n=4)	3.7%
	16c	0.77 (n=4)	1.7%	0.86 (n=4)	0.3%
	16d	0.78 (n=4)	5.9%	0.89 (n=4)	6.2%
	17	0.80 (n=4)	2.0%	0.88 (n=4)	0.9%
	18	0.79 (n=4)	3.1%	0.91 (n=4)	2.1%
	20	0.94 (n=4)	1.6%	1.04 (n=4)	4.5%
	21	1.02 (n=4)	2.4%	1.04 (n=4)	5.2%
	24	0.77 (n=4)	7.0%	0.84 (n=4)	4.7%
	25a	0.79 (n=4)	2.2%	0.89 (n=4)	1.7%
	26	0.82 (n=4)	0.7%	0.92 (n=4)	3.3%
CH	2	0.84 (n=4)	4.8%	0.85 (n=4)	2.7%
	3	0.81 (n=4)	3.1%	0.88 (n=3)**	3.2%
	5b	0.76 (n=4)	4.9%	0.80 (n=4)	2.0%
	9	0.87 (n=4)	2.2%	0.88 (n=4)	3.9%
	15	0.80 (n=4)	5.7%	0.91 (n=3)**	6.4%
	19a	0.80 (n=4)	3.5%	0.88 (n=4)	0.7%
	19b	0.84 (n=4)	3.7%	NT	
	22	0.81 (n=4)	3.6%	0.95 (n=4)	1.8%
	23	0.81 (n=4)	5.6%	0.84 (n=4)	5.2%
	25b	0.77 (n=4)	5.2%	0.83 (n=4)	5.8%
APTT	7a	0.80 (n=4)	4.6%	0.85 (n=4)	5.1%
	27	0.77 (n=4)	3.3%	0.78 (n=4)	5.8%
Overall GM		0.82 (n=34)		0.89 (n=33)	
95% CL		0.79 – 0.84		0.87 – 0.91	
Between Lab GCV		8.3%		7.4%	

NT: not tested; PT: prothrombin time; CH: chromogenic assay; APTT: activated partial thromboplastin time; N/A: not applicable; GM: geometric mean; GCV: geometric coefficient of variation; CL: confidence limits. *One assay from 4 performed was not parallel to the standard sample and has been excluded from the analysis. **One assay from the 4 performed was not linear and was excluded from the analysis.
Shaded boxes indicate outliers and are excluded from overall GM.

Table 2a: Ratios of clotting (PT) to chromogenic potency estimates for sample A relative to the 3rd IS

	PT		Chromogenic		Ratio Clotting/Chromogenic	T-Test (p)
	IU/ampoule	GCV	IU/ampoule	GCV		
FII	9.53 (n = 21)	2.9%	9.24 (n = 11)	2.2%	1.031	0.004
FX	8.29 (n = 21)	3.2%	7.81 (n = 10)	4.6%	1.061	<0.001

GCV: geometric coefficient of variation;

Table 2b: Differences in FII potency estimates for sample A by different method types

Method	n	GM IU/amp	95% LCL	95% UCL	GCV	% from the overall mean potency
PT	21	9.53	9.41	9.66	2.9%	+0.95%
APTT	2	9.61	.	.	.	+1.80%
Chromogenic	11	9.24	9.11	9.38	2.2%	-2.12%
ALL	34	9.44	9.34	9.54	3.1%	-

GM: geometric mean; GCV: geometric coefficient of variation; LCL: lower confidence limit; UCL: upper confidence limit

Table 2c: FII potency estimates for sample B against the 3rd IS for FII and X and the proposed 4th IS for FII and X, sample A (assuming the potency value of 9.44 IU/ampoule)

Putative standard	n	GM IU/amp	95% LCL	95% UCL	GCV
3rd IS	35	11.08	10.99	11.18	2.5%
Proposed 4th IS, sample A	34	11.08	10.98	11.19	2.7%

GM: geometric mean; GCV: geometric coefficient of variation; LCL: lower confidence limit; UCL: upper confidence limit

Table 2d: Differences in FX potency estimates for sample A by different method types

Method	n	GM IU/amp	95% LCL	95% UCL	GCV	% from the overall mean potency
PT	21	8.29	8.17	8.41	3.2%	+1.97%
APTT	2	8.04	.	.	.	-1.11%
Chromogenic	10	7.81	7.57	8.07	4.6%	-4.10%
ALL	33	8.13	8.00	8.26	4.6%	-

GM: geometric mean; GCV: geometric coefficient of variation; LCL: lower confidence limit; UCL: upper confidence limit

Table 2e: FX potency estimates for sample B against the 3rd IS for FII and X and the proposed 4th IS for FII and X, sample A (assuming the potency value of 8.13 IU/ampoule)

Putative standard	n	GM IU/amp	95% LCL	95% UCL	GCV
3rd IS	33	6.92	6.81	7.03	4.6%
Proposed 4th IS, sample A	33	6.91	6.84	6.99	3.0%

GM: geometric mean; GCV: geometric coefficient of variation; LCL: lower confidence limit; UCL: upper confidence limit

Table 3: Ratios of clotting (PT) to chromogenic potency estimates for sample B relative to the 3rd IS

	Potency estimates (IU/ampoule)		Ratio Clotting/Chromogenic	T-Test (p)
	PT	Chromogenic		
FII	11.06 (n = 22)	11.09 (n = 11)	0.997	0.793
FX	7.00 (n = 21)	6.76 (n = 10)	1.036	0.037

Table 4: Ratios of clotting (PT) to chromogenic potency estimates for sample P relative to the 3rd IS

	Potency estimates (IU/ampoule)		Ratio Clotting/Chromogenic	T-Test (p)
	PT	Chromogenic		
FII	0.82 (n = 22)	0.81 (n = 10)	1.012	0.667
FX	0.90 (n = 22)	0.87 (n = 9)	1.034	0.137

Figure 1a: Laboratory potency estimates (individual assays, including outliers) for FII in sample A (11/126), the proposed 4th IS Factors II and X, concentrate relative to sample S, the 3rd IS Factors II and X, concentrate, (98/590). The number in the square denotes the laboratory code.

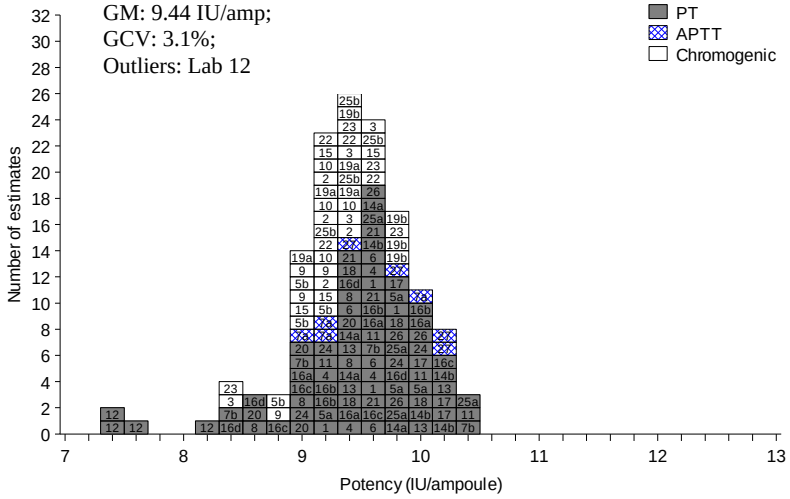


Figure 1b: Laboratory potency estimates (individual assays, including outliers) for FX in sample A (11/126), the proposed 4th IS Factors II and X, concentrate relative to sample S, the 3rd IS Factors II and X, concentrate, (98/590). The number in the square denotes the laboratory code.

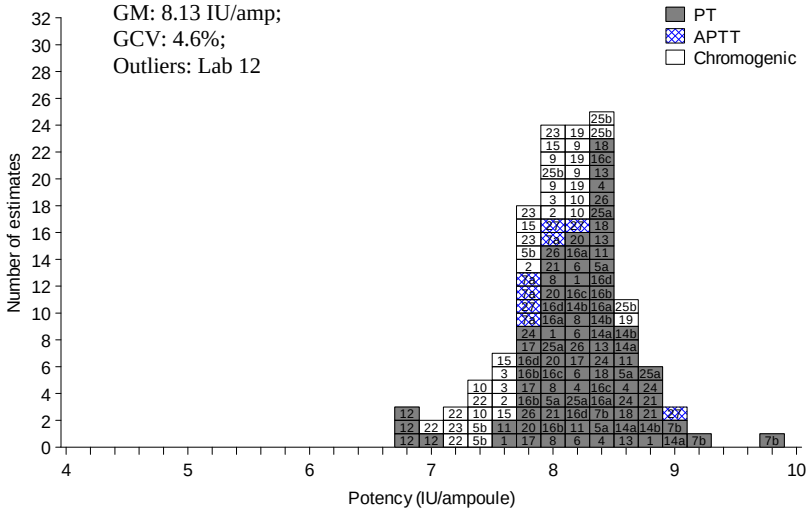


Figure 1c: Laboratory potency estimates (individual assays, including outliers) for FII in sample B (11/180), a factor II and X concentrate, relative to sample S, the 3rd IS Factors II and X, concentrate, (98/590). The number in the square denotes the laboratory code.

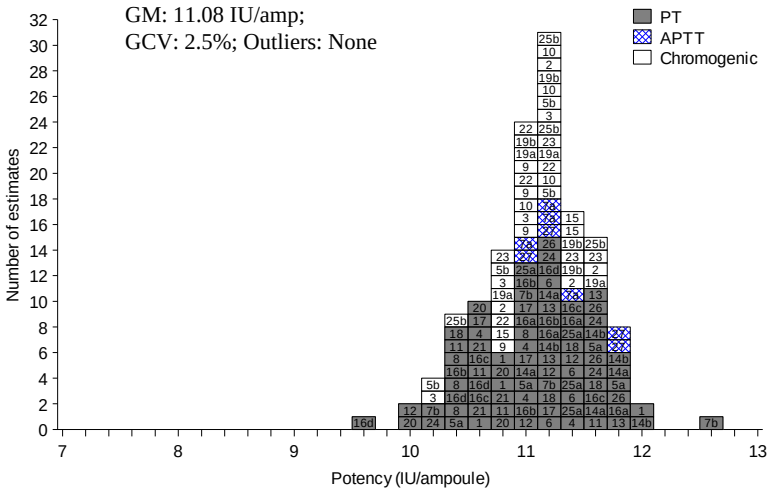


Figure 1d: Laboratory potency estimates (individual assays, including outliers) for FX in sample B (11/180), relative to sample S, the 3rd IS Factors II and X, concentrate, (98/590). The number in the square denotes the laboratory code

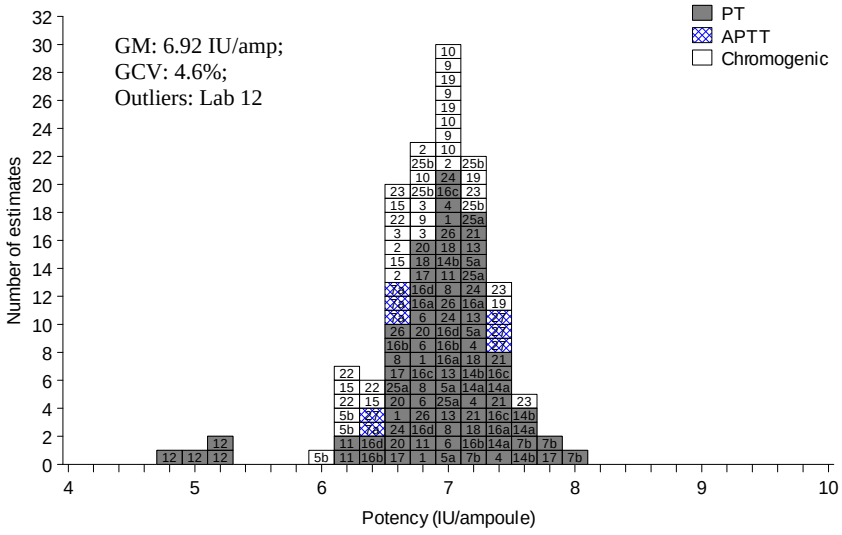


Figure 1e: Laboratory potency estimates (including outliers) for FII in sample P, the 4th IS Factors II, VII, IX, X, plasma (09/172) relative to sample S, the 3rd IS Factors II and X, concentrate, (98/590). The number in the square denotes the laboratory code.

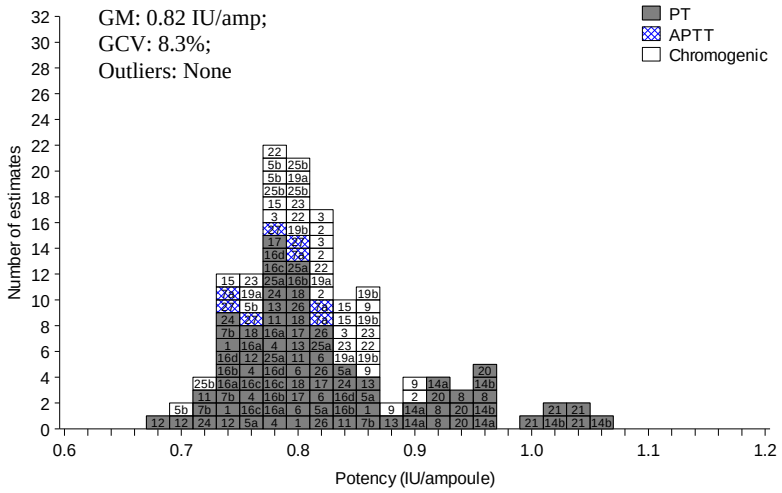
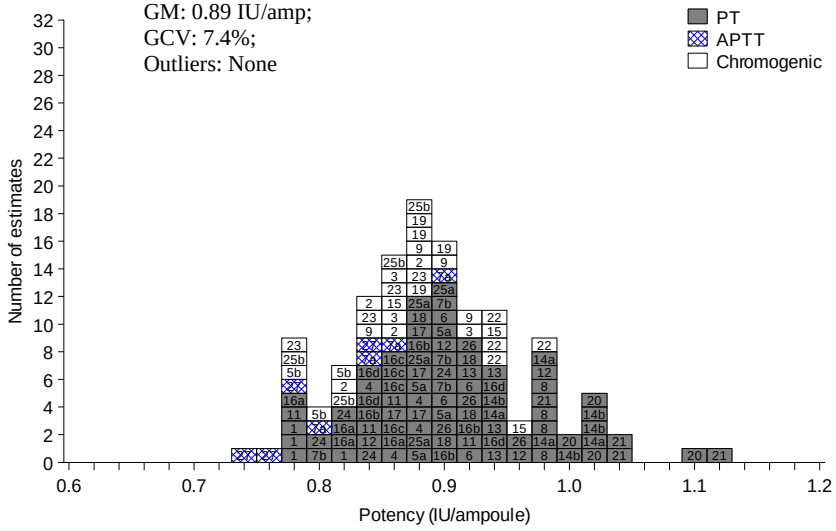


Figure 1f: Laboratory potency estimates (including outliers) for FX in sample P, the 4th IS Factors II, VII, IX, X, plasma (09/172) relative to sample S, the 3rd IS Factors II and X, concentrate, (98/590). The number in the square denotes the laboratory code.



Appendix 1: List of Participants

Renata Zadro, Clinical Hospital Center, Zagreb, Croatia

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Amanda Blande, BPL, Elstree, UK

Martina Treutlein, CSL Behring, Marburg, Germany

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Jean Amiral, Hyphen Biomed, Neuville sur Oise, France

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Mariona Bono, Diagnostic Grifols, Barcelona, Spain

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Appendix 2: Reagents, Methods and Instruments used by the Participants for FII and FX assays

Lab number	Method	Machine	Reagent	Deficient plasma
7a	**APTT**	ACL TOP 500	APTT-SP (Insturmentation Laboratory)	Precision Biologic
27		CL8 (Behnk Electronik)	Actin (Siemens)	Siemens
1	**PT**	STA compact	Neoplastin (Stago)	Stago
4		Coagrex 100S	Thromborel S (Siemens)	Sysmex
5a		BCS-XP2	Thromborel S (Siemens)	Trinity (II), Technoclone (X)
6		BCT	Neoplastin C1 (Stago)	Stago
7b		ACL TOP 500	Recombiplastin (Insturmentation Laboratory)	Precision Biologic
8		Q haemostasis analyser	DG-PT (Diagnostic Grifols)	Diagnostic Grifols
11		BCS-XP	Innovin (Siemens)	Siemens
12		ECL elite	Recombiplastin (Insturmentation Laboratory)	HTI
13		Organon Teknika MTXII	Triniclot PT (Trinity)	Trinity
14a		STAR Evolution	Neoplastin R (Stago)	Stago
14b		STAR Evolution	Neoplastin C1 (Stago)	Stago
16a		CS-1500	Innovin (Siemens)	Siemens
16b		BCS-XP	Innovin (Siemens)	Siemens
16c		CS-1500	Thromborel S (Siemens)	Siemens
16d		BCS-XP	Thromborel S (Siemens)	Siemens
17		Not given	Technoplastin His (Technoclone)	Not given
18		ACL TOP 700	Recombiplastin (Insturmentation Laboratory)	Instrumentation Laboratory
20		ACL Advance	Recombiplastin (Insturmentation Laboratory)	Pacific (II), HRF (X)
21		Stago compact	Neoplastin (Stago)	Stago
24		Sysmex CA7000	Innovin (Siemens)	Precision Biologic
25a		STA-R	Neoplastin CL5 (Stago)	Hyphen
26		BCT	Thromborel S (Siemens)	Siemens
2	**Chromogenic**	Photometer	Pentapharm	N/A
3		Anthos platereader	Pentapharm	N/A
5b		Versamax	Biophen (prothrombin and X)	N/A
9		ACL TOP	Rossix	N/A
10		STA-R	CoaChrom	N/A
15		Biomek 2000 (Beckmann)	Pentapharm	N/A
19a		Thermomax	Pentapharm (Ecarin), Rossix (RVV)	N/A
19b		Thermomax	Rossix prothrombin kit	N/A
22		BCS-XP	Ecarin and RVV – source not given	N/A
23		Spectrophotometer	Pentapharm	N/A
25b		STA-R	Biophen Hyphen kit	N/A

N/A = not applicable

Appendix 3: Individual potency estimates and geometric means (IU/amp) for each laboratory.

A. FII, A relative to S

Lab	Method	Assay 1	Assay 2	Assay 3	Assay 4	GM	GCV
7a	APTT	9.90	9.28	9.22	9.01	9.35	4.1%
27		9.76	10.19	10.27	9.34	9.88	4.5%
1	PT	9.21	9.56	9.51	9.82	9.52	2.7%
4		9.41	9.52	9.55	9.13	9.40	2.1%
5a		9.23	9.78	10.03	9.72	9.69	3.5%
6		9.57	9.61	9.54	9.43	9.54	0.8%
7b		10.47	9.66	8.35	8.99	9.34	10.1%
8		8.57	9.07	9.39	9.38	9.10	4.4%
11		10.33	9.63	9.95	9.12	9.75	5.4%
12		7.57	7.37	7.46	8.25	7.66	5.3%
13		9.92	9.41	9.33	10.24	9.72	4.5%
14a		9.83	9.41	9.43	9.59	9.56	2.0%
14b		10.17	9.94	9.58	10.21	9.97	3.0%
16a		9.49	9.53	9.07	9.92	9.50	3.7%
16b		9.13	9.55	9.16	9.93	9.44	4.1%
16c		9.67	8.95	8.82	10.19	9.39	7.0%
16d		8.47	9.79	8.63	9.50	9.08	7.4%
17		10.23	10.23	10.04	9.81	10.08	2.0%
18		9.49	9.92	9.78	9.41	9.64	2.5%
20		9.00	8.69	9.44	9.04	9.04	3.4%
21		9.52	9.50	9.57	9.30	9.47	1.3%
24		8.92	9.71	9.98	9.24	9.45	5.2%
25a		9.73	9.74	9.61	10.37	9.86	3.5%
26		9.71	9.72	9.91	9.61	9.74	1.3%
2	Chromogenic	9.44	9.14	9.15	9.18	9.23	1.6%
3		9.32	8.43	9.31	9.51	9.13	5.5%
5b		9.10	8.95	8.71	8.91	8.92	1.8%
9		8.74	9.13	9.05	9.03	8.99	2.0%
10		9.31	9.27	9.22	9.16	9.24	0.7%
15		9.17	9.01	9.63	9.16	9.24	2.9%
19a		9.47	9.31	9.14	9.08	9.25	1.9%
19b		9.71	9.85	9.77	9.36	9.67	2.3%
22		9.50	9.26	9.32	9.18	9.31	1.5%
23		9.60	9.85	9.42	8.34	9.29	7.7%
25b		9.43	9.28	9.51	9.44	9.42	1.1%

Shaded boxes represent outliers

GM: geometric mean; GCV: geometric coefficient of variation

B. FII, B relative to S

Lab	Method	Assay 1	Assay 2	Assay 3	Assay 4	GM	GCV
7a	APTT	11.33	11.15	11.02	11.18	11.17	1.2%
27		11.17	11.00	11.78	11.81	11.44	3.7%
1	PT	10.67	11.98	10.76	10.78	11.03	5.6%
4		11.48	11.03	11.05	10.63	11.04	3.2%
5a		10.47	10.91	11.64	11.78	11.19	5.7%
6		11.18	11.38	11.35	11.11	11.25	1.2%
7b		12.64	11.26	10.17	10.95	11.22	9.5%
8		10.45	10.45	11.09	10.49	10.62	2.9%
11		11.62	10.72	10.68	10.33	10.82	5.1%
12		11.03	11.21	11.35	9.96	10.87	6.2%
13		11.75	11.11	11.19	11.63	11.42	2.8%
14a		11.63	11.03	11.21	11.75	11.40	3.0%
14b		11.97	11.30	11.54	11.85	11.66	2.6%
16a		11.76	11.22	10.92	11.35	11.31	3.1%
16b		10.93	11.13	10.36	10.90	10.83	3.1%
16c		11.59	10.55	10.65	11.42	11.04	4.9%
16d		10.39	10.54	9.56	11.14	10.39	6.5%
17		11.16	11.07	11.03	10.54	10.95	2.6%
18		11.27	11.58	11.48	10.44	11.18	4.8%
20		10.82	10.10	10.73	10.58	10.55	3.1%
21		10.52	10.88	10.65	NL	10.68	1.7%
24		10.21	11.68	11.52	11.13	11.12	6.2%
25a		11.32	11.31	11.41	11.08	11.28	1.3%
26		11.71	11.61	11.54	11.16	11.50	2.1%
2	Chromogenic	11.30	11.62	10.78	11.18	11.22	3.2%
3		10.23	10.91	11.22	10.87	10.80	4.0%
5b		11.26	10.27	11.25	10.83	10.89	4.4%
9		10.98	10.88	10.91	10.97	10.93	0.4%
10		11.25	10.99	11.13	11.18	11.14	1.0%
15		NP	10.86	11.39	11.32	11.18	2.6%
19a		11.57	11.15	10.89	10.96	11.14	2.8%
19b		11.39	11.31	11.30	11.06	11.27	1.3%
22		11.23	10.87	11.05	10.96	11.03	1.4%
23		11.46	11.29	11.55	10.80	11.27	3.1%
25b		10.42	11.27	11.58	11.11	11.09	4.6%

NL – non-linear
NP – non-parallel
GM: geometric mean; GCV: geometric coefficient of variation

C. FII, P relative to S

Lab	Method	Assay 1	Assay 2	Assay 3	Assay 4	GM	GCV
7a	APTT	0.82	0.80	0.83	0.75	0.80	4.6%
27		0.76	0.74	0.80	0.78	0.77	3.3%
1	PT	0.80	0.87	0.74	0.75	0.79	7.7%
4		0.78	0.77	0.77	0.75	0.77	1.3%
5a		0.76	0.82	0.86	0.85	0.82	5.8%
6		0.81	0.81	0.82	0.82	0.81	0.8%
7b		0.86	0.73	0.73	0.75	0.77	8.1%
8		0.91	0.91	0.96	0.94	0.93	2.6%
11		0.83	0.80	0.78	0.71	0.78	6.7%
12		0.73	0.68	0.69	0.75	0.71	5.1%
13		0.88	0.80	0.79	0.86	0.83	5.2%
14a		0.97	0.89	0.90	0.91	0.92	3.7%
14b		1.01	0.95	0.97	1.07	1.00	5.1%
16a		0.77	0.77	0.74	0.77	0.76	1.9%
16b		0.79	0.84	0.74	0.80	0.79	5.6%
16c		0.78	0.75	0.76	0.78	0.77	1.7%
16d		0.78	0.84	0.73	0.77	0.78	5.9%
17		0.81	0.80	0.82	0.78	0.80	2.0%
18		0.80	0.81	0.81	0.76	0.79	3.1%
20		0.94	0.92	0.94	0.96	0.94	1.6%
21		1.03	0.99	1.02	1.05	1.02	2.4%
24		0.73	0.84	0.78	0.73	0.77	7.0%
25a		0.79	0.81	0.77	0.81	0.79	2.2%
26		0.82	0.81	0.82	0.82	0.82	0.7%
2	Chromogenic	0.90	0.82	0.81	0.83	0.84	4.8%
3		0.78	0.83	0.81	0.81	0.81	3.1%
5b		0.77	0.71	0.78	0.79	0.76	4.9%
9		0.85	0.87	0.90	0.86	0.87	2.2%
15		0.78	0.75	0.83	0.84	0.80	5.7%
19a		0.83	0.82	0.77	0.79	0.80	3.5%
19b		0.86	0.80	0.85	0.85	0.84	3.7%
22		0.85	0.82	0.80	0.79	0.81	3.6%
23		0.84	0.86	0.80	0.76	0.81	5.6%
25b		0.72	0.77	0.80	0.80	0.77	5.2%

GM: geometric mean; GCV: geometric coefficient of variation

D. FX, A relative to S

Lab	Method	Assay 1	Assay 2	Assay 3	Assay 4	GM	GCV
7a	APTT	7.91	7.76	7.78	7.80	7.81	0.9%
27		8.24	7.88	8.08	8.93	8.28	5.6%
1	PT	8.75	7.57	7.97	8.27	8.13	6.3%
4		8.47	8.14	8.63	8.46	8.43	2.5%
5a		8.35	7.96	8.45	8.61	8.34	3.4%
6		8.20	8.19	8.24	8.10	8.18	0.7%
7b		8.43	9.72	9.22	9.03	9.09	6.1%
8		7.99	8.02	8.12	7.94	8.02	0.9%
11		8.27	7.63	8.43	8.58	8.22	5.3%
12		6.76	6.82	6.77	6.91	6.81	1.0%
13		8.61	8.38	8.44	8.31	8.44	1.5%
14a		8.63	8.39	8.94	8.65	8.65	2.7%
14b		8.80	8.40	8.12	8.59	8.47	3.5%
16a		8.48	8.37	8.10	8.15	8.27	2.2%
16b		8.00	8.43	7.80	7.86	8.02	3.5%
16c		8.49	8.07	8.14	8.39	8.27	2.5%
16d		8.15	8.44	7.96	7.85	8.10	3.2%
17		7.82	8.10	7.84	7.78	7.88	1.9%
18		8.43	8.65	8.39	8.36	8.46	1.6%
20		7.90	7.91	8.07	8.23	8.03	2.0%
21		7.93	8.88	8.89	7.95	8.40	6.7%
24		8.40	8.66	8.79	7.74	8.39	5.8%
25a		8.22	7.92	8.45	8.77	8.34	4.4%
26		7.79	8.19	8.45	7.90	8.08	3.7%
2	Chromogenic	8.02	7.67	7.88	NT	7.86	2.3%
3		7.96	7.52	7.53	NL	7.67	3.3%
5b		7.42	7.32	7.76	NT	7.50	3.1%
9		8.00	8.07	8.13	8.10	8.08	0.7%
10		8.15	8.26	7.42	7.42	7.80	6.0%
15		7.65	7.92	7.64	7.72	7.73	1.7%
19a		8.54	8.24	8.28	8.28	8.33	1.7%
22		7.22	7.23	7.33	7.06	7.21	1.6%
23		7.22	8.07	7.79	7.77	7.71	4.8%
25b		7.98	8.61	8.39	8.34	8.33	3.2%

Shaded boxes represent outliers

NT – not tested
NL – non-linear

GM: geometric mean; GCV: geometric coefficient of variation

E. FX, B relative to S

Lab	Method	Assay 1	Assay 2	Assay 3	Assay 4	GM	GCV
7a	APTT	6.49	6.57	6.66	6.51	6.56	1.2%
27		7.36	6.42	7.35	7.48	7.14	7.4%
1	PT	6.72	6.57	6.71	6.95	6.74	2.4%
4		7.33	7.21	7.26	7.03	7.21	1.8%
5a		6.96	6.99	7.16	7.28	7.10	2.1%
6		7.02	6.84	6.83	6.83	6.88	1.4%
7b		7.21	7.93	7.56	7.70	7.60	4.1%
8		6.97	6.70	7.02	6.61	6.82	3.0%
11		6.75	6.27	6.94	6.27	6.55	5.3%
12		5.03	5.12	4.82	5.14	5.03	2.9%
13		7.00	6.99	7.21	7.12	7.08	1.5%
14a		7.45	7.21	7.30	7.53	7.37	2.0%
14b		7.50	7.21	7.08	7.58	7.34	3.3%
16a		7.44	7.05	7.12	6.70	7.07	4.4%
16b		7.22	6.96	6.43	6.60	6.80	5.3%
16c		7.47	6.86	7.45	7.01	7.19	4.4%
16d		6.70	7.01	6.37	6.74	6.70	4.0%
17		6.67	7.79	6.62	6.82	6.96	7.9%
18		7.11	7.12	6.99	6.88	7.02	1.6%
20		6.64	6.54	6.73	6.88	6.70	2.2%
21		7.11	7.41	7.47	7.19	7.29	2.4%
24		6.61	7.07	7.12	6.90	6.92	3.4%
25a		6.99	6.63	7.14	7.16	6.98	3.7%
26		6.74	6.90	7.07	6.64	6.83	2.8%
2	Chromogenic	6.94	6.66	6.55	6.71	6.71	2.5%
3		6.78	6.89	6.63	NL	6.77	1.9%
5b		6.23	6.14	5.98	NT	6.12	2.1%
9		6.86	6.97	6.99	6.94	6.94	0.8%
10		7.06	6.93	6.89	7.00	6.97	1.1%
15		6.36	6.70	6.25	6.61	6.48	3.3%
19a		7.37	6.96	7.07	7.29	7.17	2.7%
22		6.38	6.22	6.54	6.19	6.33	2.6%
23		7.54	7.14	7.41	6.57	7.16	6.4%
25b		7.25	6.74	6.88	7.13	7.00	3.4%

Shaded boxes represent outliers

NT – not tested
NL – non-linear

GM: geometric mean; GCV: geometric coefficient of variation

F. FX, P relative to S

Lab	Method	Assay 1	Assay 2	Assay 3	Assay 4	GM	GCV
7a	APTT	0.86	0.84	0.90	0.80	0.85	5.1%
27		0.76	0.73	0.77	0.84	0.78	5.8%
1	PT	0.81	0.78	0.78	0.78	0.79	2.4%
4		0.87	0.87	0.87	0.84	0.86	1.7%
5a		0.89	0.90	0.87	0.90	0.89	1.7%
6		0.92	0.91	0.92	0.91	0.91	0.8%
7b		0.81	0.89	0.90	0.90	0.87	5.6%
8		0.97	0.98	0.98	0.97	0.98	0.4%
11		0.93	0.86	0.84	0.78	0.85	7.4%
12		0.95	0.83	0.89	0.99	0.91	7.7%
13		0.95	0.93	0.93	0.95	0.94	1.1%
14a		0.99	0.94	1.01	0.97	0.98	3.1%
14b		1.01	0.95	1.03	1.01	1.00	3.8%
16a		0.85	0.81	0.81	0.78	0.82	3.5%
16b		0.91	0.91	0.85	0.87	0.88	3.7%
16c		0.86	0.86	0.86	0.86	0.86	0.3%
16d		0.94	0.93	0.84	0.84	0.89	6.2%
17		0.87	0.89	0.87	0.88	0.88	0.9%
18		0.89	0.92	0.93	0.89	0.91	2.1%
20		1.01	1.01	1.11	1.03	1.04	4.5%
21		1.12	1.03	0.99	1.04	1.04	5.2%
24		0.83	0.89	0.83	0.80	0.84	4.7%
25a		0.88	0.89	0.87	0.91	0.89	1.7%
26		0.90	0.91	0.97	0.92	0.92	3.3%
2	Chromogenic	0.85	0.87	0.82	0.85	0.85	2.7%
3		0.87	0.91	0.86	NL	0.88	3.2%
5b		0.80	0.78	0.81	NT	0.80	2.0%
9		0.84	0.89	0.91	0.89	0.88	3.9%
15		0.85	NL	0.94	0.95	0.91	6.4%
19a		0.88	0.88	0.88	0.89	0.88	0.7%
22		0.94	0.95	0.97	0.93	0.95	1.8%
23		0.88	0.86	0.84	0.79	0.84	5.2%
25b		0.82	0.77	0.85	0.88	0.83	5.8%

NL – Non-linear
NT – Not tested

GM: geometric mean; GCV: geometric coefficient of variation

Appendix 4: Protocol for Collaborative Study

Calibration of proposed 4th International Standard for Factors II & X, Concentrate (11/126) and Factors II & X, Concentrate (11/180).

CS471
Study Protocol

1 SAMPLES FOR ASSAY

CODE	PREPARATION
S	3rd International Standard for Factors II and X, concentrate (98/590), containing 11.2 IU FII and 10.2 IU FX per ampoule - **4 ampoules are supplied**
A	Proposed 4th International Standard for Factors II and X, concentrate (11/126), containing approximately 9 IU per ampoule FII and 8 IU per ampoule FX - **4 ampoules are supplied**
B	Factors II and X concentrate (11/180) containing approximately 11 IU FII per ampoule and 7 IU FX per ampoule - **4 ampoules supplied**.
P	4th International Standard for Factors II, VII, IX, X, plasma (07/172), containing 0.89 IU each of factors II and X - **4 ampoules supplied.**

2 STORAGE AND RECONSTITUTION OF AMPOULES OF S, A, B AND P

Store all unopened ampoules at -20°C or below. Ampoules should be allowed to warm to room temperature before reconstitution.

Directions for opening DIN ampoules
DIN ampoules have an 'easy-open' coloured stress point, where the narrow ampoule stem joins the wider ampoule body. Tap the ampoule gently to collect the material at the bottom (labelled) end. Ensure that the disposable ampoule safety breaker provided is pushed down on the stem of the ampoule and against the shoulder of the ampoule body. Hold the body of the ampoule in one hand and the disposable ampoule breaker covering the ampoule stem between the thumb and first finger of the other hand. Apply a bending force to open the ampoule at the coloured stress point, primarily using the hand holding the plastic collar.

Care should be taken to avoid cuts and projectile glass fragments that might enter the eyes, for example, by the use of suitable gloves and an eye shield. Take care that no material is lost from the ampoule and no glass falls into the ampoule. Within the ampoule is dry nitrogen gas at slightly less than atmospheric pressure. A new disposable ampoule breaker is provided with each DIN ampoule.

Reconstitute the ampoule contents by adding 1 ml of distilled water. Allow the ampoule to stand for 10 minutes at room temperature and aid reconstitution by gentle swirling. Transfer contents to a plastic tube and store at 4 °C prior to the assays.

3 ASSAY DESIGN

Four sets of ampoules are provided. Assays for factors II and X should be carried out on each of the 4 sets. Each set should be tested on a different day (see schedule below). A balanced order of testing should be used.

Day 1, ampoule set 1	**FII assay**	**S_1**	**A_1**	**B_1**	**P_1**	**P_2**	**B_2**	**A_2**	**S_2**
	FX assay	**S_1**	**A_1**	**B_1**	**P_1**	**P_2**	**B_2**	**A_2**	**S_2**
Day 2, ampoule set 2	**FII assay**	**P_1**	**S_1**	**A_1**	**B_1**	**B_2**	**A_2**	**S_2**	**P_2**
	FX assay	**P_1**	**S_1**	**A_1**	**B_1**	**B_2**	**A_2**	**S_2**	**P_2**
Day 3, ampoule set 3	**FII assay**	**B_1**	**P_1**	**S_1**	**A_1**	**A_2**	**S_2**	**P_2**	**B_2**
	FX assay	**B_1**	**P_1**	**S_1**	**A_1**	**A_2**	**S_2**	**P_2**	**B_2**
Day 4, ampoule set 4	**FII assay**	**A_1**	**B_1**	**P_1**	**S_1**	**S_2**	**P_2**	**B_2**	**A_2**
	FX assay	**A_1**	**B_1**	**P_1**	**S_1**	**S_2**	**P_2**	**B_2**	**A_2**

Each letter refers to a set of three different dilutions (e.g. 1/10, 1/20, 1/40) and **A1, A2** and **S1, S2** etc. refer to separate sets of dilutions (replicates) made independently from the same ampoule. The range of dilutions should be chosen to lie on the most linear portion of the dose-response relationship.

Please include a pre-dilution step in the appropriate factor-deficient plasma, unless your assay has been validated for use without a pre-dilution in plasma. The same range of dilutions should be used for all three materials (**S, A, B, P**). The assays should be completed within two hours of reconstitution. It is preferable for the whole study to be carried out over four days.

4 RESULTS

Raw data (e.g. clotting times) should be recorded on the results sheet. You are also invited to calculate the relative potencies of A, B and P vs S from your own assay results using the assigned potencies of S given in section 1. Please return your raw data and calculated potency estimates by **12th January 2012** to:

helen.barson@nibsc.hpa.org.uk

Dr H Barson, Biotherapeutics Group, NIBSC, Blanche Lane, South Mimms, Potters Bar, Hertfordshire, United Kingdom, EN6 3QG, Fax: +44 (0) 1707 641050

Appendix 5: Draft Instruction for Use (IFU) for the Proposed 4th International Standard for Blood Coagulation Factors II and X, Concentrate 11/126

NIBSC
A centre of the Health Protection Agency

Health Protection Agency

WHO International Standard
The 4th IS for Blood Coagulation Factors II and X Concentrate
NIBSC code: 11/126
Instructions for use
(Version 1.00, Dated)

1. INTENDED USE

The 4th International Standard for Factors II and X, Concentrate, consists of ampoules, coded 11/126, containing aliquots of a freeze-dried concentrate prepared from human plasma. This preparation was established as the 4th International Standard for Factors II and X, Concentrate, by the Expert Committee on Biological Standardisation of the World Health Organisation in October 2012. This preparation is intended for use as a calibrant for FII and X potency estimates in Factor IX and prothrombin complex oncentrates

2. CAUTION

This preparation is not for administration to humans.

The preparation contains material of human origin, and either the final product or the source materials, from which it is derived, have been tested and found negative for HBsAg, anti-HIV and HCV RNA. As with all materials of biological origin, this preparation should be regarded as potentially hazardous to health. It should be used and discarded according to your own laboratory's safety procedures. Such safety procedures should include the wearing of protective gloves and avoiding the generation of aerosols. Care should be exercised in opening ampoules or vials, to avoid cuts.

3. UNITAGE

The standard was calibrated by 28 laboratories in 14 countries against the 3rd International Standard for Factors II and X, Concentrate (98/590). Functional assays performed were based on snake venom chromogenic methods, prothrombin time clotting assays and APTT clotting assays, using the appropriate clotting factor deficient plasma (Factor II or Factor X deficient plasma). The assigned potencies are as follows:

Factor II: 9.4 IU/ampoule
Factor X: 8.1 IU/ampoule

4. CONTENTS

Country of origin of biological material: United Kingdom.
The single batch of material was diluted in 40 mM Tris, 120 mM NaCl, 1.6 mg/ml trehalose and 4 mg/ml human serum albumin, pH 7.4. The material was distributed in glass ampoules, filled and freeze-dried according to guidelines for production of international standards (1). All material had been tested and was found negative for anti-HIV1/2, HBsAg and anti-hepatitis C. The mean weight of liquid content of 587 check weight ampoules was 1.0074g, with a coefficient of variation of 0.14%. The mean weight of the freeze-dried plug was 25.5 mg, with a coefficient of variation of 0.84%. the mean residual moisture was 0.69%.

5. STORAGE

Unused material must be discarded and not frozen for later use. Unopened ampoules should be stored at or below -20°C.
Please note: because of the inherent stability of lyophilized material, NIBSC may ship these materials at ambient temperature.

6. DIRECTIONS FOR OPENING

DIN ampoules have an 'easy-open' coloured stress point, where the narrow ampoule stem joins the wider ampoule body.
Tap the ampoule gently to collect the material at the bottom (labeled) end. Ensure that the disposable ampoule safety breaker provided is pushed down on the stem of the ampoule and against the shoulder of the ampoule body. Hold the body of the ampoule in one hand and the disposable ampoule breaker covering the ampoule stem between the thumb and first finger of the other hand. Apply a bending force to open the ampoule at the coloured stress point, primarily using the hand holding the plastic collar.
Care should be taken to avoid cuts and projectile glass fragments that might enter the eyes, for example, by the use of suitable gloves and an eye shield. Take care that no material is lost from the ampoule and no glass falls into the ampoule. Within the ampoule is dry nitrogen gas at slightly less than atmospheric pressure. A new disposable ampoule breaker is provided with each DIN ampoule.

7. USE OF MATERIAL

No attempt should be made to weigh out any portion of the freeze-dried material prior to reconstitution.

Allow ampoules to warm to room temperature. Open ampoule, taking care to ensure that all material is in the lower part, and reconstitute with 1.0 ml distilled water. The reconstituted Standard should be used as soon as possible.

8. STABILITY

Reference materials are held at NIBSC within assured, temperature-controlled storage facilities. Reference Materials should be stored on receipt as indicated on the label.

NIBSC follows the policy of WHO with respect to its reference materials.

It is the policy of WHO not to assign expiry dates to international reference materials. Accelerated degradation studies have shown that the 4th International Standard is very stable in unopened ampoules stored at –20°C. The predicted loss of activity is <0.01% of the original potency per year for both Factors II and X when stored at –20°C.

9. REFERENCES

Campbell PJ. Procedures used for the production of biological standards and reference preparations. J Biol Standardisation. 1974, 2, 259-267.

10. ACKNOWLEDGEMENTS

We acknowledge all participants in the international collaborative study. We are grateful to BioProducts Laboratory Ltd, Grifols Biologicals Inc., Baxter AG for supply of candidate materials for the pilot and definitive collaborative studies.

11. FURTHER INFORMATION

Further information can be obtained as follows;
This material: enquiries@nibsc.hpa.org.uk
WHO Biological Standards:
http://www.who.int/biologicals/en/
JCTLM Higher order reference materials:
http://www.bipm.org/en/committees/jc/jctlm/
Derivation of International Units:
http://www.nibsc.ac.uk/products/biological_reference_materials/frequently_asked_questions/how_are_international_units.aspx
Ordering standards from NIBSC:
http://www.nibsc.ac.uk/products/ordering_information/frequently_asked_questions.aspx
NIBSC Terms & Conditions:
http://www.nibsc.ac.uk/terms_and_conditions.aspx

12. CUSTOMER FEEDBACK

Customers are encouraged to provide feedback on the suitability or use of the material provided or other aspects of our service. Please send any comments to enquiries@nibsc.hpa.org.uk

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Health Protection Agency

13. CITATION

In all publications, including data sheets, in which this material is referenced, it is important that the preparation's title, its status, the NIBSC code number, and the name and address of NIBSC are cited and cited correctly.

14. MATERIAL SAFETY SHEET

Physical and Chemical properties			
Physical appearance: White solid		Corrosive:	No
Stable:	Yes	Oxidising:	No
Hygroscopic:	Yes	Irritant:	Yes
Flammable:	No	Handling:	See caution, Section 2
Other (specify):	Contains material of human origin		

Toxicological properties	
Effects of inhalation:	Not established, avoid inhalation
Effects of ingestion:	Not established, avoid ingestion
Effects of skin absorption:	Not established, avoid contact with skin

Suggested First Aid	
Inhalation:	Seek medical advice
Ingestion:	Seek medical advice
Contact with eyes:	Wash with copious amounts of water. Seek medical advice
Contact with skin:	Wash thoroughly with water.

Action on Spillage and Method of Disposal
Spillage of ampoule contents should be taken up with absorbent material wetted with an appropriate disinfectant. Rinse area with an appropriate disinfectant followed by water. Absorbent materials used to treat spillage should be treated as biological waste.

15. LIABILITY AND LOSS

Information provided by the Institute is given after the exercise of all reasonable care and skill in its compilation, preparation and issue, but it is provided without liability to the Recipient in its application and use.

It is the responsibility of the Recipient to determine the appropriateness of the standards or reference materials supplied by the Institute to the Recipient ("the Goods") for the proposed application and ensure that it has the necessary technical skills to determine that they are appropriate. Results obtained from the Goods are likely to be dependant on conditions of use by the Recipient and the variability of materials beyond the control of the Institute.

All warranties are excluded to the fullest extent permitted by law, including without limitaton that the Goods are free from infectious agents or that the supply of Goods will not infringe any rights of any third party.

The Institute shall not be liable to the Recipient for any economic loss whether direct or indirect, which arise in connection with this agreement.

The total liability of the Institute in connection with this agreement, whether for negligence or breach of contract or otherwise, shall in no event exceed 120% of any price paid or payable by the Recipient for the supply of the Goods.

If any of the Goods supplied by the Institute should prove not to meet their specification when stored and used correctly (and provided that the Recipient has returned the Goods to the Institute together with written notification of such alleged defect within seven days of the time when the Recipient discovers or ought to have discovered the defect), the Institute shall either replace the Goods or, at its sole option, refund the handling charge provided that performance of either one of the above options shall constitute an entire discharge of the Institute's liability under this Condition.

16. INFORMATION FOR CUSTOMS USE ONLY

Country of origin for customs purposes*: United Kingdom * Defined as the country where the goods have been produced and/or sufficiently processed to be classed as originating from the country of supply, for example a change of state such as freeze-drying.
Net weight: ~25 mg
Toxicity Statement: Toxicity not assessed
Veterinary certificate or other statement if applicable. **Attached**: No

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