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An international collaborative study to value assign the WHO 2nd International Standard for Factor VII concentrate (10/252)

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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 addressed to the World Health Organization, 1211 Geneva 27, Switzerland, attention: Quality Safety and Standards (QSS). Comments may also be submitted electronically to the Responsible Officer: Dr Ana Padilla at email: padillaa@who.int

Summary
An international collaborative study was organized to calibrate a replacement for the WHO 1st International Standard for Factor VII concentrate (97/592). The study involved 24 laboratories from 11 different countries representing manufacturers, clinical and regulatory groups. Laboratories were asked to measure the FVII content of two freeze-dried candidate materials: sample A (10/250) and B (10/252), using clotting and/or chromogenic methods. For each laboratory, potencies were calculated for A and B relative to S, the 1st IS, and the geometric mean potency was calculated for each of the candidates independently for clotting and chromogenic methods. Statistical analysis revealed potencies determined by clotting and chromogenic methods are significantly different for both candidates. In addition there was a significant assay method bias within the clotting results for candidate A, with laboratories using recombinant thromboplastin reagent producing lower potencies compared to those using a natural purified thromboplastin. For candidate B there was no method bias observed, however the potency determined by clotting methods was significantly higher than the chromogenic potency. Clotting methods for FVII potency are sensitive to the amount of activated FVII (FVIIa) present whereas chromogenic methods are not. Candidate B was found to contain higher levels of FVIIa compared to candidate A and this may explain the relatively higher potency for candidate B determined by clotting methods. Because of the assay method bias observed with Candidate A and recombinant thromboplastin reagents, and the larger discrepancy between clotting and chromogenic methods for Candidate A, it is proposed that candidate B (10/252) should be established as the WHO 2nd IS for factor FVII concentrate. The difference between the potency estimates by the different methods is too large to reconcile. It is therefore proposed that 10/252 is established with a potency of 9.8 IU for chromogenic methods and 10.6 IU for clotting methods.

Introduction
The 1st International Standard (IS) for factor VII concentrate (97/592) was established by the Expert Committee on Biological Standardisation (ECBS) of the World Health Organization (WHO) in October 1998[1]. A potency of 6.3 IU was assigned to the preparation by clotting and chromogenic methods, relative to the 2nd IS factors II, VII, IX, X plasma (94/746) and normal plasma pools. This reference material is used for potency assignment to human coagulation FVII concentrate preparations used to treat FVII deficiency, and for FVII-containing prothrombin complexes used for reversal of anticoagulant treatment.

Stocks of the current IS are almost exhausted and a replacement is needed. An international collaborative study was therefore organised to calibrate a replacement IS for FVII concentrate, and the outcome of this study is described in this report. A final assessment of the stability of the 1st IS (97/592) is presented in Appendix A, which shows the material is highly stable with no measurable loss of potency for ampoules kept under normal storage conditions (-20 °C).

Materials
Candidate materials were provided by two manufacturers: Baxter Bioscience, Vienna, Austria and LFB, Lille, France. Following small scale trial fills, definitive fills were carried out on both materials and coded 10/250 and 10/252 (designated respectively as candidate preparations A and B in the collaborative study). Both candidate materials were provided as freeze-dried preparations of Factor VII purified from pooled human plasma that had been tested and found negative for HBsAg, HIV antibody, HCV antibody and HCV RNA by PCR. The material was reconstituted and diluted to a final concentration of 10 IU/ml (based on the labelled potency) in 40 mM Tris buffer (pH 7.4) containing 0.12 M sodium chloride, 4 mg/ml

trehalose and 10 mg/ml human albumin. 5 ml DIN ampoules were filled with 1 ml aliquots of the diluted material and lyophilised following WHO procedures. Filling and lyophilisation was carried out at NIBSC and the ampoules are stored at NIBSC (Potters Bar, UK) at -20 °C. Further details of the lyophilisation records for 10/250 and 10/252 are given in the table below.

	A (10/250)	**B (10/252)**
Date of fill	17 February 2011	24 February 2011
Number of containers	4846	6873
Mean fill mass g (cv %)	1.0083 (0.1343) n=205	1.007 (0.155) n=274
	Samples taken from pumps 1, 2 and 3 throughout the fill	
Mean dry weight g (cv %)	0.0372 (2.43) n=6	0.0297 (1.55) n=6
Mean residual moisture % (cv %)	0.083 (22.07) n=12	0.097 (23.89) n=12
Mean oxygen head space % (cv %)	0.37 (29.66) n=12	0.39 (33.75) n=12

Participants

An invitation to participate in the collaborative study was posted on the website of the Standardisation Subcommittee (SSC) of the International Society on Thrombosis and Haemostasis (ISTH) to maximise publicity and attract participants worldwide. The invitation was also distributed to laboratories throughout the OMCL (Official Medicines Control Laboratories) network in Europe. Manufacturers of therapeutic concentrates and diagnostic kits, and other regulatory laboratories, were also approached directly. A total of 24 laboratories agreed to participate and all completed the study and returned results. Participating laboratories represented 11 different countries (Germany (3), UK (2), USA (2), France (6), Austria (4), Netherlands, Spain (2), Croatia, Japan, S. Korea, Australia) and of these 8 were regulatory, 15 from industry and 1 clinical laboratory. A list of the participants is provided in the acknowledgements section; however the order of listing does not correspond to the laboratory code number.

Assay methods and study design

The aim of the study was to assign Factor VII potency values to candidate materials A and B relative to the WHO 1st IS for Factor FVII, concentrate (97/592) (sample S in the study) using chromogenic and clotting methods. The WHO 4th IS Factors II, VII, IX and X plasma (09/172) was also included in the study as sample P for information only to compare the assay of plasma vs. concentrate.

Kits for measuring Factor VII potency by the chromogenic assay are commercially available based on the same two-stage principle via activation of Factor X to Xa. One-stage clotting assays are used with commercially available Factor VII-deficient plasma and thromboplastin reagent (tissue factor) from various sources.

Study participants were provided with four ampoules of samples S, A, B and P and were requested to carry out at least four Factor VII assays across two separate days or sessions, using fresh ampoules for each assay. Laboratories were instructed to include all four preparations (S, A, B and P) in each of the four assays with a minimum of three dilutions of each preparation, including two or more replicates of each dilution within each assay. An

approximate Factor VII content of 10 IU per ampoule for samples A and B was given to enable an appropriate dilution regime.

Participants were requested to return the raw data from their assays to NIBSC. All data analysis was performed at NIBSC, although participants were invited to provide their own potency estimates if they wished. Participants were also requested to provide information about the assay method and equipment used to obtain the results.

In addition a subset of laboratories with experience of measuring activated Factor VII (FVIIa) were provided with four additional ampoules of samples A, B and S and invited to measure FVIIa content of each using their routine method and usual FVIIa standard. Each laboratory was requested to perform four independent assays and return FVIIa potency estimates for each sample including confidence limits.

Statistical analysis

Relative potencies and confidence limits for samples A, B and P were calculated relative to sample S for each assay by parallel-line analysis[2] using the software program Combistats[3]. Deviations from linearity and parallelism were considered significant at the 5% level ($p<0.05$). 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.

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 ($GCV = \{10^{s}-1\}\times 100\%$ where s is the standard deviation of the log transformed potency potencies).

Comparisons between assay methods have been made by unpaired two-tailed t-tests of log transformed laboratory mean potencies.

Results

FVII potency estimates for candidate preparations

A summary of potency estimates is shown in Table 1 (clotting) and Table 2 (chromogenic) for candidate A (10/250) and Table 3 (clotting) and Table 4 (chromogenic) for candidate B (10/252). An overall potency estimate was calculated for each candidate material independently for clotting and chromogenic methods.

Potency estimates for clotting methods were calculated from the results from 80 independent assays performed by 17 different laboratories. Six different thromboplastin reagents were represented in the study. Laboratory 21 performed 4 independent sets of assays on 2 different analysers with 2 different thromboplastin reagents. These results were analysed independently and coded as 21a-21d. A summary of the thromboplastin reagents used in the study is shown below.

Number of laboratories	Thromboplastin reagent	Source
7	Thromborel S (Siemens Healthcare Diagnostics)	Human placental

4	Neoplastin plus (Diagnostica Stago)	Rabbit brain
1	Thromboplastin HIS (Technoclone)	
1	DG-PT (Diagnostic Grifols)	
4	Innovin (Siemens Healthcare Diagnostics)	Human recombinant
3	Recombiplastin 2G (Instrumentation Laboratory)	

Potency estimates for chromogenic methods were calculated from the results from 44 independent assays performed by 11 different laboratories. Two different commercial assay kits were used in the study with 7 laboratories using the Coaset FVII kit from Chromogenix and 2 laboratories using the Biophen VII kit (Chromogenic VII) from Hyphen Biomed. Two laboratories used an in-house method.

Candidate A

The distribution of results for candidate A for each laboratory by assay method is shown in Figure 1. Potency estimates by clotting methods ranged from 7.08 to 9.79 IU with a mean of 8.65 IU and GCV of 9.6 %. Potency estimates by chromogenic methods ranged from 9.22 to 10.73 IU with a mean of 9.96 IU and GCV of 3.8 %. The potency estimates obtained by clotting and chromogenic methods were compared by unpaired two-tailed t-test of log transformed laboratory geometric means, and found to be significantly different ($p<0.001$).

Within the results for clotting assays shown in Figure 1(I), there appears to be two distinct groups with human recombinant thromboplastin reagents producing lower potencies than natural purified thromboplastin (from rabbit brain and human placenta). The results of laboratories using recombinant and purified thromboplastin were compared by unpaired two-tailed t-test and found to be significantly different ($p<0.001$).

Candidate B

The distribution of results for candidate B for each laboratory by assay method is shown in Figure 2. Potency estimates by clotting methods ranged from 8.75 to 12.80 IU with a mean of 10.57 IU and GCV of 8.3 %. Potency estimates by chromogenic methods ranged from 9.21 to 10.67 IU with a mean of 9.78 IU and GCV of 4.6 %. The potency estimates obtained by clotting and chromogenic methods were found to be significantly different ($p=0.006$).

The results for clotting assays shown in Figure 2(I) appear to be randomly distributed with no bias observed with different thromboplastin reagents. This observation was confirmed as no significant difference was found comparing the results of laboratories using recombinant and purified thromboplastin ($p=0.488$).

FVII potency estimates for sample P

A summary of potency estimates for sample P is shown in Table 5 for clotting methods and Table 6 for chromogenic methods. The distribution of results for each laboratory by assay method is shown in Figure 3. Potency estimates by clotting methods ranged from 0.87 to 1.61 IU with a mean of 1.19 IU and GCV of 18.7 %. Potency estimates by chromogenic methods ranged from 0.86 to 0.96 IU with a mean of 0.91 IU and GCV of 3.6 %. The results from assays 2 and 4 from laboratory 2 were excluded due to significant deviations from parallelism. The difference in potency estimates between clotting and chromogenic methods highlights a method discrepancy when measuring plasma samples relative to a concentrate standard. This method discrepancy and the large inter-laboratory variation for the clotting assays validate the need for a separate concentrate and plasma standards to observe the principle of measuring 'like vs. like' materials.

Activated FVII (FVIIa) content of candidate preparations

Potency estimates of FVIIa in samples A, B and S are shown in Table 7, and the overall results summarised below:

Sample	Estimate IU/ampoule	Lower 95% confidence limit	Upper 95% confidence limit	GCV	N
A	0.39	0.37	0.42	8.1 %	8
B	1.29	1.16	1.44	14.0 %	8
S	0.42	0.39	0.45	10.0 %	8

The result from laboratory 4 was excluded from the final calculation as a statistical outlier, and the result from laboratory 19 was excluded as sample S was used as the standard.

Discussion

The aim of this study was to calibrate a replacement for the WHO 1st IS for FVII concentrate suitable for potency assignment to FVII concentrate preparations using clotting and chromogenic methods, and to ensure continuity of the IU. Two candidates were prepared from therapeutic preparations of FVII concentrate (received as donations from different manufacturers) by dilution and lyophilisation. Both candidates fulfilled criteria with regard to precision and homogeneity of fill, residual moisture content and headspace oxygen.

Potency estimates were obtained in the study for both candidates by clotting and chromogenic methods relative to the 1st IS. All results returned by participating laboratories were statistically valid and all data were included in the final calculations. The clotting results had a higher inter-laboratory variation than the chromogenic results as represented by the GCV% (9.6 and 8.3 for A and B by clotting methods; 3.8 and 4.6 by chromogenic methods). The difference in variability may be explained by the small number of different kits used for the chromogenic assays compared to the wide range of different reagents used for clotting assays. For both candidates potency estimates obtained by the different methods were significantly different, with the potency by clotting methods for candidate A being 13.1 % lower than the chromogenic potency. For candidate B the difference was smaller however the trend was reversed with a clotting potency 8.1 % higher than the chromogenic result.

The higher potency for candidate B by clotting methods could be explained by higher levels of activated factor FVII (FVIIa), and this was consistent with the results obtained in the study by laboratories measuring FVIIa content by direct methods. The FVIIa potency estimate for candidate B in the study was 1.29 IU, which was higher than both candidate A (0.39 IU) and the current IS (0.42 IU). Clotting methods are widely known to be sensitive to FVIIa levels where chromogenic methods are not. The relatively lower potency estimate for candidate A with clotting methods is unlikely to be explained by FVIIa levels alone, and may be caused by the assay reagent bias observed in the study, where laboratories using recombinant thromboplastin returned significantly lower potencies than those using natural purified thromboplastin, and chromogenic methods. The underlying cause of this reagent bias is not clear, but it appears to be specific to candidate A. No bias was found with candidate B or with sample P (the 4th IS Factors II, VII, IX and X plasma). For the calibration of the 1st IS (97/592) thromboplastin reagents from the same range of sources (rabbit brain, human placental and human recombinant) were used, however for this study potency assignment was

relative to human plasma. No reagent bias was reported for this study at the time, and based on present findings with sample P (also human plasma) no bias would be expected.

Proposal

Based on the larger difference in potency estimates between clotting and chromogenic results for candidate A, and the apparent assay bias when recombinant thromboplastin reagent is used, candidate B is proposed as the replacement IS. The difference between the potency estimates by the different methods is too large to reconcile and it would not be statistically valid to assign a single potency for all methods. Furthermore a combined potency for all methods would result in a shift in potency values assigned to therapeutic concentrates which could affect patient dosing.

It is therefore proposed that candidate B (10/252) should be established as the WHO 2nd IS for Factor VII concentrate with a potency of 9.8 IU for chromogenic methods and 10.6 IU for clotting methods.

Participants' response

All participants that have so far responded to the report (23/24) agreed with the conclusion in the report that preparation 10/252 (candidate B in the study) be proposed as the WHO 2nd International Standard for Factor VII Concentrate with a potency of 9.8 IU for chromogenic methods and 10.6 IU for clotting methods.

Laboratory 10 agreed that "the statistically significant difference between the results by clotting and chromogenic methods justifies the assignment of two different potency values for this IS" and therefore "fully supports the proposal stated in the report". Laboratory 19 also commented: "I am not surprised to see differences between thromboplastins and assay methods for FVII. Most clotting methods are sensitive to trace amounts of Factor VIIa (overestimated) whilst chromogenic assays are not".

Laboratory 9 identified minor typographical errors, and identified the thromboplastin reagent used by them in the study was DG-PT and not Technoplastin HIS, however both are sourced from rabbit brain so the conclusions are unaffected. The report has been updated to reflect this change and typographical errors corrected.

Expert review by the Factor VIII and Factor IX Subcommittee of the SSC/ISTH

Expert opinion was sought from a panel of experienced scientists selected by the Chair of the Factor VIII and Factor IX Subcommittee of the SSC of the ISTH. Responses were received from 16 experts and all agreed with the proposals.

Several reviewers commented on the issue of activated Factor VII (FVIIa) being present in the standard and "whether the amount of activated factor VII in a standard can be standardized" and "how the preparation of these standards might be altered to reduce FVII activation in advance of the assignment of potency of WHO 3rd IS".

Standardizing or reducing the amount of FVIIa in the standard would only be effective if the level of FVIIa in all FVII concentrate products was also standardized or reduced, and this is only important when clotting methods are used as chromogenic methods are not sensitive to the level of FVIIa. The material used to prepare the proposed 2nd IS was sourced from a

product manufacturer and is therefore representative of the products for which it is intended to be used. A more appropriate solution to the FVIIa issue for future replacement IS may be to restrict its use for potency assignment to chromogenic methods, or provide a potency by clotting methods for information only.

Another reviewer wondered "if the loss of the difference between results with different thromboplastin reagents with candidate B also relates in some way to FVIIa content – perhaps this can be explored before preparation of the 3rd IS?"

This is an interesting point and could easily be investigated. It seems counter-intuitive at first since the 1st IS and Candidate A share similar levels of FVIIa, and it may therefore be expected that any reagent bias would be associated with differences in FVIIa content. It may however be true that the higher level of FVIIa in Candidate B in some way compensates for the differences between the different thromboplastin reagents. If this is true, then it provides another argument against deliberately reducing the FVIIa content of future replacement IS, and supports the use of chromogenic methods exclusively.

Another reviewer commented on the summary section of the report, that "it could be more clearly explained why candidate B was chosen, i.e. the difference of the potency estimates between the clotting and chromogenic assays was smaller in candidate B than it was in candidate A and in candidate B there was no assay bias using recombinant thromboplastin reagents. As it is stated now the suggestion is made that candidate B was chosen because of the higher content of activated factor VII". The summary has now been amended to state the reasoning behind the choice more clearly as it is stated in the proposals later in the report.

Stability of the proposed candidate material 10/252

Long term stability

Predictions for the long term stability of the proposed candidate material (10/252) will be assessed over time by monitoring the Factor VII potency of ampoules stored under accelerated degradation conditions. Ampoules of the proposed candidate preparation were stored at a range of temperatures immediately following lyophilisation (-150 °C, -70 °C, -20 °C, +4 °C, +20 °C, +37 °C, +45 °C and +56 °C).

To provide an indication of long term stability, two ampoules of 10/252 from each storage temperature were assayed following storage under accelerated degradation conditions for one year. Potency estimates were obtained for the degradation samples (+20 °C, +37 °C, +45 °C and +56 °C) relative to ampoules stored at -20 °C, using clotting and chromogenic methods at NIBSC. The results are presented in the table below, expressed as a percentage of the potency result for the -20 °C samples. Each result is based on a combined potency from two ampoules assayed separately in duplicate.

Stability data of FVII concentrate candidate B (10/252) after 1 year storage at elevated temperatures				
Assay method	% activity relative to -20 °C			
	+20 °C	+37 °C	+45 °C	+56 °C
Clotting	99.6 %	88.1 %	79.7 %	14.9 %
Chromogenic	100.4 %	86.1 %	77.6 %	9.9 %

The results suggest that candidate material 10/252 is very stable, with little or no observed loss of potency when stored up +20 °C. The results were successfully fitted to the Arrhenius Equation and the prediction for the % loss of activity per year for samples stored at -20 °C (normal storage conditions) was 0.000 %. Stability monitoring is ongoing and will be assessed for longer time periods at lower storage temperatures.

Bench stability following reconstitution

To provide an indication of the stability of the proposed candidate material throughout a typical assay period, the potency of the candidate B (10/252) was monitored following reconstitution. A reference was prepared by pooling several ampoules and freezing small aliquots which were stored at -80 °C until required. On the day of the study several ampoules were reconstituted, pooled and divided into two stoppered plastic tubes, labelled T1 and T2. Each sample was stored on melting ice and assayed for FVII activity relative to the frozen reference at time 0, 1 hour, 2 hours and 4 hours. This process was carried out independently for clotting and chromogenic methods, with all assays carried out at NIBSC. The FVII potencies are provided in the table below, based on two assays combined and expressed as a percentage of the frozen reference relative to time zero.

% activity relative to fresh ampoule at time zero (95 % confidence interval)			
Assay method	1 hours	2 hours	4 hours
Clotting	93.8 % (85.8 – 100.8)	90.0 % (76.5 – 106.1)	89.4 % (78.1 – 93.8)
Chromogenic	97.3 % (93.4 – 101.3)	100.4 % (72.3 – 139.4)	97.7 % (97.5 – 98.0)

This data supports a recommendation that potency assays should be completed within 2 hours of reconstitution of the standard, and the IFU (instructions for use) will reflect this. It will however be recommended that end users investigate stability following reconstitution for their own storage and assay conditions.

References

[1] Hubbard AR, Rigsby P, Barrowcliffe T. Report on an international collaborative study to calibrate the 1st International Standard for factor FVII concentrate. Ref: BS/98.1876. WHO Expert Committee on Biological Standardisation; 1998.

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[3] Daas, A. (2008). CombiStats v4.0, www.combistats.eu, *EDQM, Council of Europe*.

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The Factor VIII and IX SSC Subcommittee of the ISTH.

All participants who took part in the study (listed below), and to everyone else involved who is not identified personally.

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Table 1. FVII potency estimates for Candidate A (IU per ampoule) from clotting assays for each laboratory relative to Sample S. Potencies and geometric coefficient of variation (GCV %) are calculated from the geometric mean results of all valid assays returned from each laboratory. The overall geometric mean is also shown, together with the GCV % of laboratory means.

Laboratory number	Number of assays	Candidate A geometric mean FVII potencies (IU per ampoule)	GCV %	Geometric mean	GCV %
1	4	8.13	14.1	8.65	9.6
3	4	7.08	1.9		
4	4	7.77	6.6		
6	4	8.98	1.7		
7	4	7.69	16.7		
9	4	8.78	2.5		
11	4	8.30	9.7		
12	4	9.31	2.9		
14	4	9.77	3.8		
15	4	9.18	0.8		
16	4	9.00	4.6		
17	4	7.58	5.4		
19	4	9.04	3.2		
20	4	8.91	5.5		
21a	4	8.73	8.1		
21b	4	7.84	3.9		
21c	4	9.27	6.7		
21d	4	8.97	6.0		
22	4	9.63	3.2		
23	4	9.79	4.5		
	Total = 80				

Table 2. FVII potency estimates for Candidate A (IU per ampoule) from chromogenic assays for each laboratory relative to Sample S. Potencies and geometric coefficient of variation (GCV %) are calculated from the geometric mean results of all valid assays returned from each laboratory. The overall geometric mean is also shown, together with the GCV % of laboratory means.

Laboratory number	Number of assays	Candidate A geometric mean FVII potencies (IU per ampoule)	GCV %	Geometric mean	GCV %
1	4	9.98	4.3	9.96	3.8
2	4	10.08	2.2		
3	4	10.09	6.4		
5	4	10.18	5.3		
8	4	10.73	1.5		
10	4	9.59	3.3		
13	4	9.94	0.9		
14	4	9.95	1.2		
18	4	9.84	5.6		
19	4	9.22	4.1		
24	4	10.03	10.2		
	Total = 44				

Figure 1. Mean FVII potencies of Candidate A (IU per ampoule) from clotting assays (I) and chromogenic assays (II) for each laboratory relative to Sample S. Columns represent the number of laboratories with results in the corresponding FVII concentration range. Each box represents an individual laboratory, with shading identifying the thromboplastin reagent used (I).

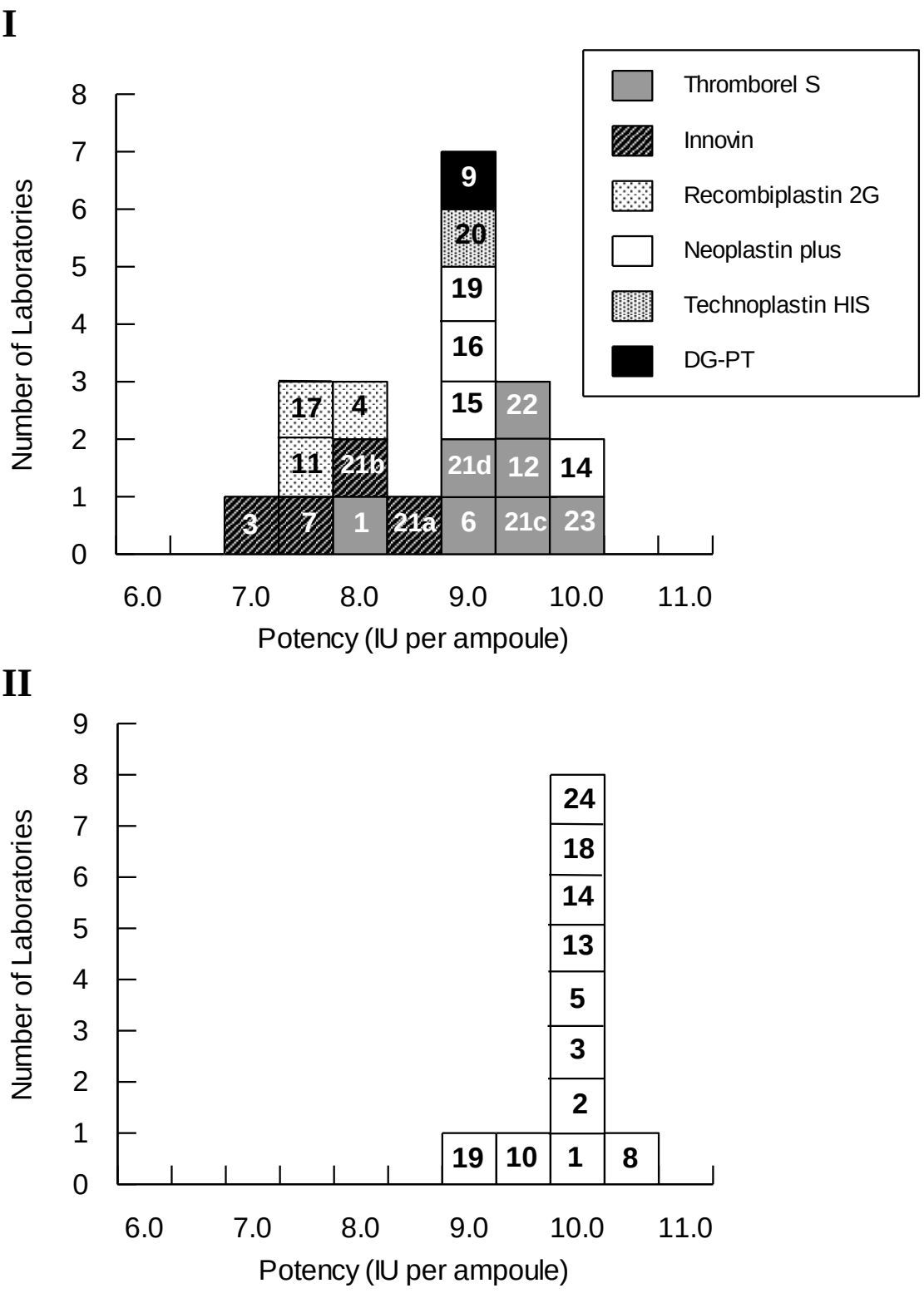


Table 3. FVII potency estimates for Candidate B (IU per ampoule) from clotting assays for each laboratory relative to Sample S. Potencies and geometric coefficient of variation (GCV %) are calculated from the geometric mean results of all valid assays returned from each laboratory. The overall geometric mean is also shown, together with the GCV % of laboratory means.

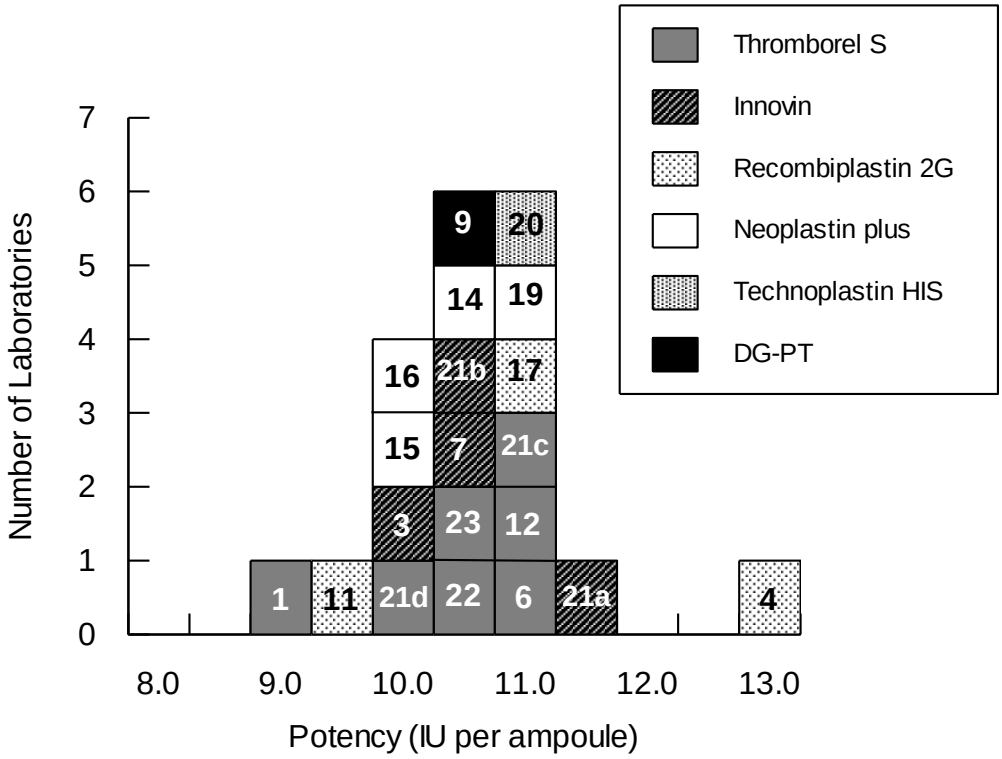
Laboratory number	Number of assays	Candidate B geometric mean FVII potencies (IU per ampoule)	GCV %	Geometric mean	GCV %
1	4	8.75	14.0	10.57	8.3
3	4	9.86	2.8		
4	4	12.80	9.2		
6	4	11.12	1.6		
7	4	10.64	7.0		
9	4	10.66	2.5		
11	4	9.37	14.6		
12	4	11.14	1.7		
14	4	10.31	0.4		
15	4	9.96	4.9		
16	4	10.19	8.0		
17	4	10.82	8.4		
19	4	10.86	1.9		
20	4	11.22	4.6		
21a	4	11.34	6.3		
21b	4	10.78	4.9		
21c	4	11.19	4.4		
21d	4	9.96	12.0		
22	4	10.73	3.9		
23	4	10.33	2.2		
	Total = 80				

Table 4. FVII potency estimates for Candidate B (IU per ampoule) from chromogenic assays for each laboratory relative to Sample S. Potencies and geometric coefficient of variation (GCV %) are calculated from the geometric mean results of all valid assays returned from each laboratory. The overall geometric mean is also shown, together with the GCV % of laboratory means.

Laboratory number	Number of assays	Candidate B geometric mean FVII potencies (IU per ampoule)	GCV %	Geometric mean	GCV %
1	4	10.31	7.3	9.78	4.5
2	4	9.58	1.5		
3	4	9.48	6.8		
5	4	10.16	5.0		
8	4	10.67	3.2		
10	4	9.45	2.3		
13	4	9.88	3.3		
14	4	9.89	2.1		
18	4	9.21	4.1		
19	4	9.42	6.1		
24	4	9.61	10.0		
	Total = 44				

Figure 2. Mean FVII potencies of Candidate B (IU per ampoule) from clotting assays (I) and chromogenic assays (II) for each laboratory relative to Sample S. Columns represent the number of laboratories with results in the corresponding FVII concentration range. Each box represents an individual laboratory, with shading identifying the thromboplastin reagent used (I).

I



II

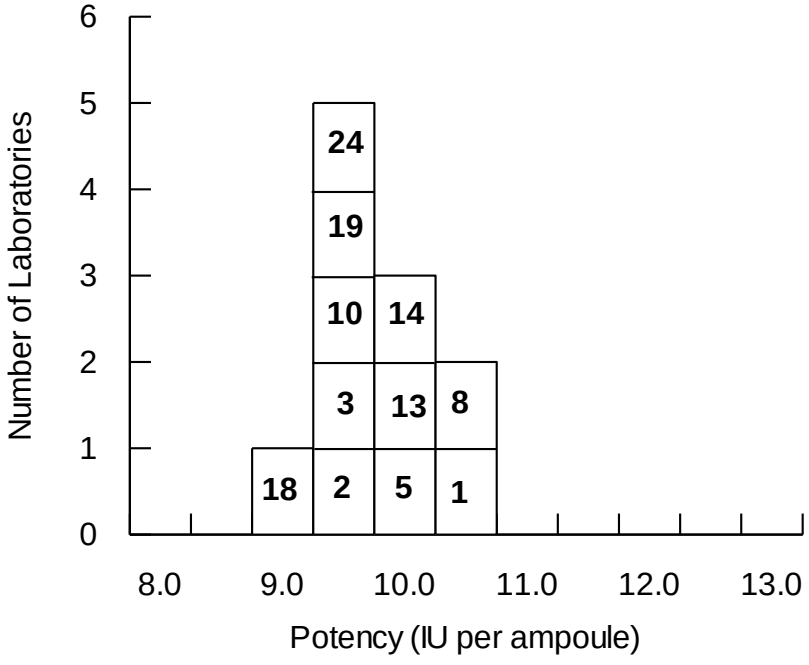


Table 5. FVII potency estimates for Sample P (IU per ampoule) from clotting assays for each laboratory relative to Sample S. Potencies and geometric coefficient of variation (GCV %) are calculated from the geometric mean results of all valid assays returned from each laboratory. The overall geometric mean is also shown, together with the GCV % of laboratory means.

Laboratory number	Number of assays	Candidate P geometric mean FVII potencies (IU per ampoule)	GCV %	Geometric mean	GCV %
1	4	1.20	7.6	1.19	18.7
3	4	1.37	3.7		
4	4	1.02	14.8		
6	4	1.05	2.3		
7	4	1.56	12.8		
9	4	1.22	6.7		
11	4	1.35	4.0		
12	4	1.03	0.9		
14	4	0.87	6.3		
15	4	1.08	3.1		
16	4	1.13	7.8		
17	4	1.11	12.3		
19	4	0.94	5.7		
20	4	0.98	8.4		
21a	4	1.51	7.7		
21b	4	1.30	2.5		
21c	4	1.61	4.3		
21d	4	1.24	7.0		
22	4	1.26	4.4		
23	4	1.36	3.9		
	Total = 80				

Table 6. FVII potency estimates for Sample P (IU per ampoule) from chromogenic assays for each laboratory relative to Sample S. Potencies and geometric coefficient of variation (GCV %) are calculated from the geometric mean results of all valid assays returned from each laboratory. The overall geometric mean is also shown, together with the GCV % of laboratory means.

Laboratory number	Number of assays	Candidate P geometric mean FVII potencies (IU per ampoule)	GCV %	Geometric mean	GCV %
1	4	0.89	18.6	0.91	3.6
2	2	0.96	1.0		
3	4	0.86	3.3		
5	4	0.93	3.3		
8	4	0.93	5.0		
10	4	0.92	2.8		
13	4	0.89	2.6		
14	4	0.89	3.6		
18	4	0.96	4.6		
19	4	0.91	3.9		
24	4	0.90	5.8		
	Total = 42				

Figure 3. Mean FVII potencies of Sample P (IU per ampoule) from clotting assays (I) and chromogenic assays (II) for each laboratory relative to Sample S. Columns represent the number of laboratories with results in the corresponding FVII concentration range. Each box represents an individual laboratory, with shading identifying the assay method (I).

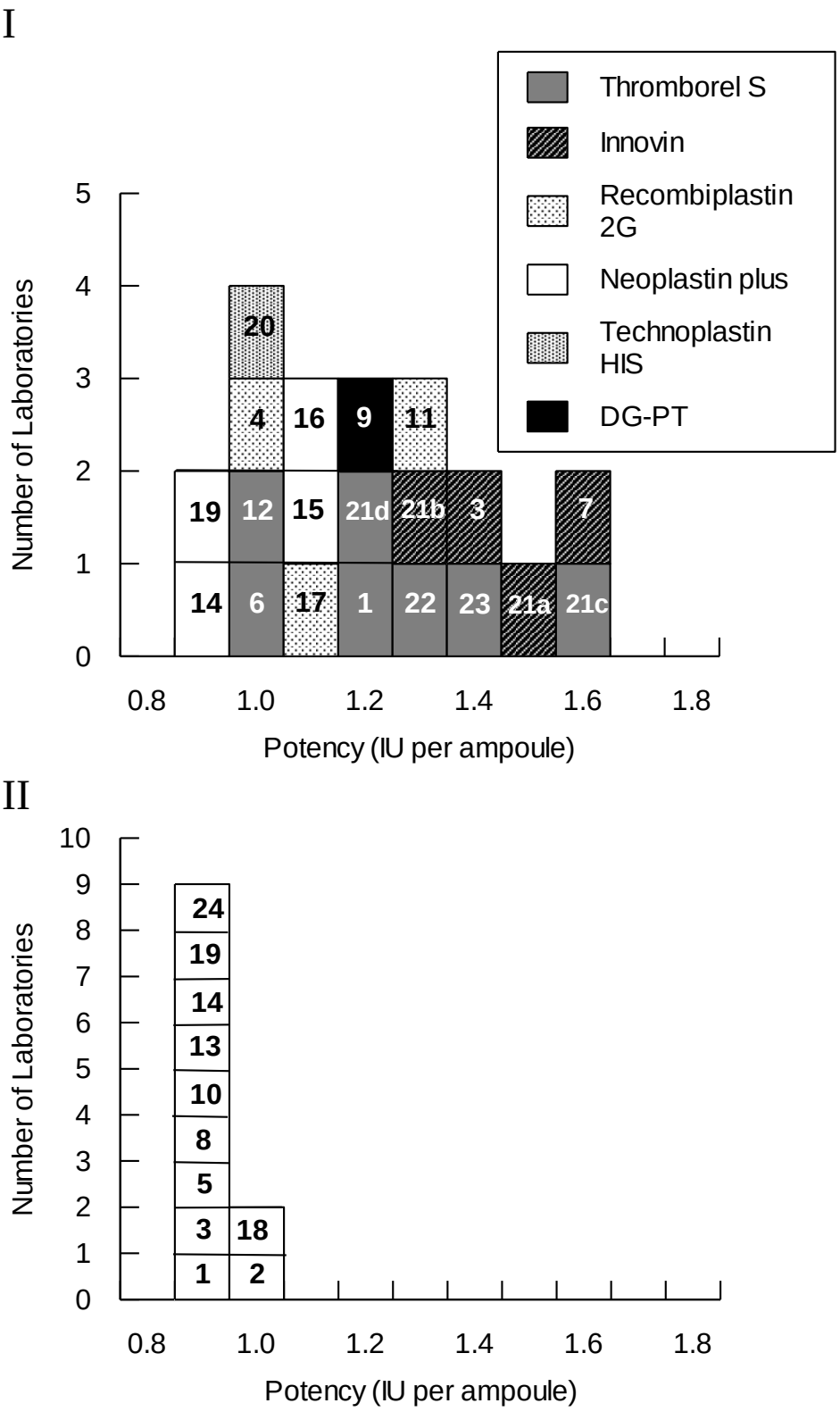


Table 7. Laboratory estimates of FVIIa potencies for Samples A, B and S (IU per ampoule) . Potency estimates, confidence limits and geometric coefficient of variation (GCV %) were calculated from the geometric mean of results returned from each laboratory. The overall geometric mean is also shown, together with the GCV % of laboratory means.

Laboratory number	Sample	n	Estimate	Lower 95% confidence limit	Upper 95% confidence limit	GCV %	Geometric mean	GCV %
1	A	4	0.36	0.32	0.40	7.8	0.39	8.1
2		4	0.44	0.37	0.53	12.5		
3		4	0.38	0.33	0.43	9.3		
4*		4	1.72	0.59	5.00	95.5		
13		4	0.40	0.40	0.40	0.0		
14		4	0.41	0.38	0.45	5.0		
15		4	0.36	0.29	0.45	14.5		
16		4	0.41	0.34	0.50	13.5		
17		4	0.36	0.33	0.39	5.2		
19*		4	0.14	0.13	0.15	4.2		
1	B	4	1.27	0.97	1.65	18.1	1.29	14.0
2		4	1.45	1.39	1.52	2.9		
3		4	1.20	1.02	1.40	10.7		
4*		4	4.51	2.17	9.35	58.2		
13		4	1.29	1.03	1.62	15.2		
14		4	1.64	1.43	1.88	8.9		
15		4	1.06	0.80	1.41	19.3		
16		4	1.21	0.92	1.59	18.7		
17		4	1.29	1.08	1.55	12.2		
19*		4	0.18	0.17	0.20	5.3		
1	S	4	0.43	0.36	0.51	12.1	0.42	10.0
2		4	0.43	0.40	0.46	4.5		
3		8	0.49	0.44	0.53	11.6		
4*		3	1.44	0.77	2.67	28.3		
13		4	0.40	0.40	0.40	0.0		
14		4	0.41	0.37	0.45	6.5		
15		4	0.35	0.29	0.41	11.7		
16		4	0.43	0.38	0.49	8.5		
17		4	0.41	0.39	0.44	4.3		

*results excluded from final calculation

Appendix A.
An assessment of the stability of the 1st IS for FVII concentrate (97/592).

Five independent samples of the 1st IS for FVII concentrate stored at -150, -20, +4 and +20 °C for 13 years and 4 months (11 September 1998 – 6 January 2012) were assayed by the one-stage FVII clotting method, and by the FVII chromogenic method, at NIBSC. The results are presented in the table below for the higher temperatures as a % of the mean result of ampoules stored at -150 °C.

Assay number	Potencies expressed as a % of ampoules stored at -150 °C					
	Clotting method			Chromogenic method		
	-20	+4	+20	-20	+4	+20
1	98.690	104.560	86.241	95.731	89.471	73.280
2	98.602	99.289	89.217	100.442	93.288	82.401
3	94.506	91.708	86.151	103.374	95.884	76.822
4	100.556	100.316	86.962	102.918	97.439	75.081
5	99.489	98.942	90.626	94.609	87.211	77.015
Geometric mean	98.346	98.874	87.821	99.348	92.578	76.86
GCV %	2.39	4.86	2.28	4.18	4.76	4.48
95 % confidence interval	95.504 - 101.274	93.215 - 104.877	85.398 - 90.313	94.423 - 104.53	87.388 - 98.077	72.792 - 81.155

These results indicate that the 97/592 is very stable at -20 °C, the normal storage temperature for this material, with no measurable loss of potency by clotting or chromogenic assay methods. Even ampoules stored at ambient temperature (+20 °C) only lost between 13 and 24 % of activity. This result provides confidence in the continuity of the IU for a replacement IS calibrated relative to the 1st IS.

Appendix B.
Draft instructions for use (IFU) for the proposed WHO 2nd IS for Factor VII Concentrate (10/252).

WHO International Standard
2nd International Standard for Factor VII Concentrate
NIBSC code: 10/252
Instructions for use
(Version 1.00, Dated)

1. INTENDED USE
The 2nd International Standard for Factor VII Concentrate (10/252) was established by Expert Committee on Biological Standardisation of the World Health Organisation in October 2012. The intended use of this preparation is for potency estimation of Factor VII concentrates.

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 potency of the 2nd International Standard for Factor VII Concentrate (10/252) was determined relative to the 1st International Standard for Factor VII Concentrate (97/592) in an international collaborative study involving 24 laboratories representing 11 different countries. Different potency values were assigned according to the assay method used:

The potency by chromogenic methods is 9.8 IU per ampoule.

The potency by clotting methods is 10.6 IU per ampoule.

4. CONTENTS
Country of origin of biological material: United Kingdom.
The bulk material used to prepare the 2nd International Standard was donated by one manufacturer as a freeze-dried preparation of Factor VII purified from pooled human plasma. The material was reconstituted and diluted in 40 mM Tris buffer (pH 7.4) containing 0.12 M sodium chloride, 4 mg/ml trehalose and 10 mg/ml human albumin to a final concentration of 10 IU/ml (based on the labelled potency). A total of 6873 5 ml DIN ampoules were filled with 1 ml aliquots of the diluted material , with a mean filling weight of 1.007 g (cv = 0.155 %). Freeze-drying was done following WHO procedures to produce ampoules with a mean dry weight of 0.0297 g (cv = 1.55 %) and a residual moisture of 0.097 % (cv = 23.89 %).

5. STORAGE
Upon receipt unopened ampoules should be stored in the dark 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 the ampoule to reach ambient temperature before opening and reconstitute with 1.0 ml distilled water, using gentle shaking. Transfer the contents to a plastic tube and keep on ice.

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 an expiry date to their international reference materials and they remain valid with the assigned potency until withdrawn or amended.

Predicitons on long term stability are made by monitoring ampoules stored under accelerated degradation conditions over time.

Based on the results of a stability test it is advised that ampoules are stored on wet ice following reconstitution and potency assays should be completed within 2 hours of reconstitution.

9. REFERENCES
A report of the collaborative study to calibrate the standard is available from WHO, reference number WHO/BS/2012.XXXX

10. ACKNOWLEDGEMENTS
We are grateful to all the participants that took part in the collaborative study, to Baxter Bioscience (Austria) and LFB (France) for the supply of candidate materials from which the international standard was selected, and to the Factor VIII and Factor IX Subcommittee of the Standardization and Scientific Committee (SSC) of the International Society on Thrombosis and Haemostasis (ISTH)

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

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A World Health Organization Laboratory for Biological Standards

NIBSC
A centre of the Health Protection Agency

Health Protection Agency

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

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 powder		Corrosive:	No
Stable:	Yes	Oxidising:	No
Hygroscopic:	Yes	Irritant:	No
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 limitation 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: 29.7 mg
Toxicity Statement: Non-toxic
Veterinary certificate or other statement if applicable. **Attached:** No

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