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**A Collaborative Study to Establish
The 3rd International Standard for Antithrombin, Concentrate, Human**

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SUMMARY

Twenty-one laboratories participated in a collaborative study to establish a replacement for the 2nd International Standard for Antithrombin, Concentrate, Human (96/520). There was excellent agreement between laboratories, as indicated by low inter-laboratory variability (% GCVs) for the 3 candidate materials which consisted of one recombinant and two plasma derived clinical concentrates. In terms of performance, stability profiles and physical characteristics of the candidates, all 3 materials are very similar. However, there is a larger number of ampoules of sample C, 06/166 available. It is therefore proposed that sample C, 06/166 is considered as the 3rd International Standard for Antithrombin, Concentrate, Human, with labelled potencies for both functional activity (**4.4 IU/ampoule**) and antigenic value (**4.5 IU/ampoule**). All participants and experts from the Scientific and Standardisation Subcommittee (SSC) of the International Society on Thrombosis and Haemostasis (ISTH) have agreed with this proposal.

INTRODUCTION

Antithrombin is the most important endogenous inhibitor of activated coagulation factors. Antithrombin deficiency, whether congenital or acquired, results in an increased risk of venous thromboembolism. Replacement therapy using clinical antithrombin concentrates is available and a reliable potency standard is essential for accurate value assignment of these clinical products. The 2nd International Standard (IS) for Antithrombin, Concentrate, Human, 96/520 was established in 1997¹ and its value assigned against the 1st International Standard for Antithrombin, Concentrate, 88/548. Due to the depletion of stock of 96/520, a replacement is required. In the present study, three candidate materials were assayed against the 2nd International Standard for Antithrombin, Concentrate, 96/520, with a view to establishing a new material as the 3rd International Standard for Antithrombin, Concentrate, Human. The 2nd International Standard for Antithrombin, Plasma, Human, 93/768 was also included in this study to compare the antithrombin unitage as defined by the concentrate and plasma standards.

PARTICIPANTS

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. A total of twenty-one laboratories including 9 antithrombin concentrate producers, 8 regulatory control laboratories, 3 diagnostic manufacturers and 1 clinical laboratory returned results.

SAMPLES

Coded samples included in the study were:

A - the 2nd International Standard for Antithrombin, Concentrate, Human, 96/520. Functional potency: 4.7 IU/ampoule; Antigen value: 5.1 IU/ampoule.

B - a purified recombinant human antithrombin concentrate, 06/160. Potency range for both function and antigen: 3.0 - 5.0 IU/ampoule.

C - a purified plasma derived human antithrombin concentrate, 06/166. Potency range for both function and antigen: 3.0 - 5.0 IU/ampoule.

D- a purified plasma derived human antithrombin concentrate, 06/168. Potency range for both function and antigen: 3.0 - 5.0 IU/ampoule.

P - the 2nd International Standard for Antithrombin, Plasma, Human, 93/768. Potency for both function and antigen: 0.85 IU/ampoule.

ASSAY METHODS

Each participant was requested to perform their routine in-house method(s) for antithrombin, including at least one type of functional assay. All the functional assays performed were based on the heparin co-factor chromogenic method. Seventeen laboratories used thrombin, 3 laboratories used FXa and 1 laboratory (Lab 16) returned functional data for 2 different types of heparin co-factor assays, one with thrombin and the other with factor Xa and these are referred to as 16 and 16a. Nine laboratories performed antigenic assays. Lab 14 returned results from 2 different types of antigen methods which are referred to as 14 and 14a. Of the laboratories that performed antigenic methods, 2 laboratories performed Laurell, 4 laboratories employed rate nephelometry, 3 laboratories used ELISA and 1 laboratory used immunoturbidimetric assay. Two laboratories returned one set of data each for crossed immunoelectrophoresis (CIE). A list of methods performed by the participants is given in Appendix 2.

STUDY DESIGN

Participants were requested to perform four independent assays for each type of method. They were requested to assay concurrently a series of at least three dilutions of each of the five study samples. The assay order of the materials (including replicates) was varied to give an overall balanced order of testing. Duplicate measurements on the same dilutions could be included if so wished. Participants were requested to return raw assay data, along with their own estimates for the antithrombin potency of materials B, C, D and P using A as the standard.

RESULTS

Assay Data

Functional activity assays were performed by 20 laboratories. In total, 85 functional assays were considered for analysis as each laboratory performed 4 assays apart from lab 16 which performed two types of functional activity assay and Lab 1 repeated one assay. Antigen assays were performed by 9 laboratories. In total, 40 antigen assays were analysed. For the functional activity assays, some laboratories also included an in-house or commercial calibrant but as these were not included as part of the study design, results for these samples were not considered in the analysis.

Statistical Analysis

In the majority of cases, parallel line analysis² was employed to calculate the potencies of samples B, C, D and P relative to A. After an appropriate transformation, the response was plotted against log dose, assessed visually and formally tested for deviations against linearity or parallelism at the 1% level of significance. For the majority of laboratories a squared transformation yielded the best linearity in functional assays. For Lab 14 and 15 the untransformed responses gave the best linearity. For Lab 6, responses were transformed to percentages relative to the estimated upper and lower limits of the dose-response curve and weighted regression of logit response on log dose was used. For antigen assays by Lab 6, 12, 13, 16 and 21, a log transform was used and for Lab 14, 14a and 18 no transformation was required.

For Lab 2, 5 and 11 functional assays no transformation which linearised the dose response curve could be found so the dose response curve was split into two sections which were analysed separately. A geometric mean of the two estimates was then calculated as the overall potency estimate for each assay. In each case, the two estimates were not discrepant by more than 10% indicating satisfactory parallelism.

Lab 8 only tested sample A at multiple doses in their functional assay. Samples B, C, D, and P were repeated at single doses and the mean responses were read from the dose response curve of sample A to produce potency estimates. While this did not allow an assessment of parallelism to be made, the results were in agreement with other laboratories and have been included in the overall analysis.

Using all results considered acceptable, potency estimates have been combined as unweighted geometric means. Variability within-labs and between-labs has been expressed using geometric coefficients of variation (GCV)³. In addition, each of the laboratories reported their own potency estimates for each assay and a geometric mean and GCV have been calculated based on their reported potency estimates.

To test for any outlier results, an analysis of variance of log potencies with Duncan's multiple comparison test was used⁴. Any laboratory that was significantly different to all others at the 1% level of inference was classified as an outlier.

Assay Validity

Satisfactory linearity and parallelism was achieved in the majority of assays. For the functional assays by Lab 16a, 16b, 21, 9 (assay 4 only) and the antigen assays by Lab 12 and 16, the appearance of the dose response curve was acceptable but significant deviations from linearity and/or parallelism were detected due to small residual error resulting from small differences in the replicate responses. Exclusion of these assays did not change the overall geometric mean potency estimates of the samples by more than one percent and the results were included in all further calculations.

For the antigen assays by Lab 2 and 19, there were no dose-response curves and the laboratories' own potency estimates have been used for analysis.

For the functional assays, an incomplete set of results was obtained for some laboratories and these are as indicated below:

Lab 1: The second assay of sample D was found to be non-linear and was independently retested in a separate assay but the variability amongst replicates was large and the dose response curves were rejected. The fourth assay was completely rejected as the lab noted a possible error with the machine.

Lab 7: Each assay was split over two plates, with four plates testing A, B & C and four plates testing A, D & P. For assays testing A, B & C, one was rejected due to the level of variability in the responses. In the others, C was non-parallel to B and A. For assays testing A, D & P, A was non-linear and non-parallel to D and P in one assay. In another, P was non-parallel to A and D.

Lab 9: Sample D was non-linear in assay 4, therefore excluded.

Lab 11: Sample P was not tested.

Lab 14: Sample C was non-linear in assay 2, therefore excluded.

Lab 15: Sample P in assay 1 excluded due to large differences between replicates.

Lab 16: Sample P was non-linear, therefore excluded.

Lab 19: Sample P non-linear in assay 2, therefore excluded.

Functional Activity

Detailed values of individual assay and geometric mean potencies for samples B, C, D and P relative to sample A, the 2nd IS for Antithrombin, Concentrate, Human, calculated for each laboratory by NIBSC are shown in table 1.

Summaries of geometric mean potencies from individual laboratories and the overall geometric mean potencies and 95% confidence intervals (CI) for the NIBSC calculation only are listed in tables 2 – 5. The potencies according to laboratories' own calculations are also indicated. The data are also shown in histogram form for samples B, C, D and P for the NIBSC calculations in figures 1 – 4.

The histograms illustrate good agreement between laboratories for samples B, C, D and P. However, for sample B, Lab 16a was identified as an outlier and the results were calculated including and excluding this outlier. In addition, the spread is greatest for sample P suggesting that agreement is slightly better for samples B, C and D.

The variability within each laboratory, expressed as geometric coefficients of variation (GCV's) for each sample is given in tables 2 - 5 for those based on NIBSC potency calculations. With the exception of Lab 7 that obtained GCVs greater than 20% for samples B and D, all laboratories obtained GCV less than 6% (the majority being less than 4%). There was no apparent difference in intra-laboratory variability between methods.

Variability between laboratories for the potency estimates of samples B, C and P relative to sample A, are also shown in tables 2 – 5 as % GCV's using the NIBSC estimated potencies. The %GCVs for samples B (excluding Lab 16a), C, D and P are 5.0, 3.2, 3.3 and 8.0 respectively.

Lab 4, 10, 16a, and 19 performed FXa based assays whilst all other labs performed thrombin based assays. For each sample a t-test of the log potency estimates showed no significant differences between the two methods.

Antigen Measurement

Detailed values of individual assay and geometric mean potencies for samples B, C, D and P relative to sample A, the 2nd IS for Antithrombin, Concentrate, Human, calculated for each laboratory by NIBSC are shown in table 6.

Summaries of geometric mean potencies from individual laboratories and the overall geometric mean potencies with 95% confidence intervals (CI) for the NIBSC calculation only are listed in tables 7 – 10. The potencies according to laboratories' own calculations are also indicated. The data are also shown in histogram form for samples B, C, D and P for the NIBSC calculations in figures 5 - 8.

Both intra- and inter-laboratory variability are slightly higher for antigen measurement than for functional activity. With the exception of Lab 14 that obtained over 30% intra-laboratory GCV, the GCVs for other laboratories ranged from 0.6% to 10.8%. The inter-laboratory GCVs for sample B, C, D and P (excluding Lab 6) are 4.7, 9.9, 10.8 and 6.8 respectively. The main cause of the higher inter-laboratory GCV for samples C and D by comparison with sample B is due to the higher estimation of values for samples C and D by Lab 6, the results of which were included as they were not statistically classified as outliers.

Comparison with locally calculated results

The estimates calculated at NIBSC were compared to those reported by the laboratories. Lab 2, 20 and 21 used an in-house reference to calculate their own potency estimates so did not calculate samples B, C, D and P directly against A. These labs also provided potency estimates for sample A and the lab geometric mean potency estimates for functional activity were 5.06, 5.34 and 4.97 IU/ampoule for Lab 2, 20 and 21 respectively. In all cases this is higher than the assigned potency of 4.7 IU/ampoule. Because of this, the NIBSC potency estimates for these labs are more discrepant than for other labs. NIBSC estimates for all samples are consistently around 88% of the Lab 21 estimates and 94% of the Lab 20 estimates. For Lab 2 the results are less discrepant but still slightly lower for samples B, C and D. In almost all other cases where no samples or assays have been excluded the NIBSC results are within 3% of the reported results. This also suggests that the calibrants or in-house standards used by these 3 laboratories should be reassessed for comparability with the IS.

Overall potency estimates relative to the 2nd International Standard for Antithrombin, Concentrate, Human, 96/520

The overall mean potency estimates for all the samples are summarised in table 11. For sample B, the potency estimates are 4.26 and 4.35 IU/ampoule for function and antigen respectively. For sample C, the potency estimates are 4.40 and 4.45 IU/ampoule for function and antigen respectively. For sample D, the potency estimates are 4.38 and 4.35 IU/ampoule for function and antigen respectively.

The assigned values for sample P, the 2nd IS for Antithrombin, Plasma, Human, 93/768 are 0.85 IU/ampoule for both function and antigen. The potency estimates from the current study are 0.84 and 0.79 IU/ampoule for function and antigen respectively. The difference between the assigned and estimated potency for functional activity is 1.2%. Given that the GCV for the current study is 8.0% and the 95% CI is 0.81 – 0.87 IU/ampoule, this result indicates that there is good continuity of the plasma and concentrate international unit of functional activity for antithrombin. For the antigen measurement, there is 7.1 % difference in the potency estimate when compared with the assigned value. However, considering that the range of values (0.75 to 0.93 IU/ampoule) is relatively wide and that the measurement of concentrate against plasma does not always give good comparative responses, more data would be needed to confirm whether the 7% difference requires further attention.

Crossed immunoelectrophoresis (CIE)

Crossed immunoelectrophoresis (CIE) is a semi-quantitative technique and is used qualitatively by many manufacturers to assess their products. It is part of the European Pharmacopoeia (EP) Monograph for Antithrombin Concentrate, the requirement being 60% of the product must be heparin binding. Since only 2 laboratories (Lab 9 and 14) performed this method and only one set of data was returned by each laboratory, it was not possible to carry out any statistical analysis. However, there was agreement for samples B, C, D and P, but not for sample A. No low affinity material was detected in samples B, C, D and P. For sample A, the 2nd IS, Lab 9 found the sample contained only high affinity material, while Lab 14 reported 87.5% of high affinity fraction.

STABILITY STUDY

Preliminary accelerated degradation data on samples B, C and D indicate satisfactory stability. After 9 months storage of samples B, C and 6 months storage of sample D, NIBSC has found no significant loss of functional activity in the samples stored at +20, +37 and 45°C (activity relative to the –150°C samples). Further accelerated and real time degradation studies will be carried out. WHO and users of this material will be informed of any significant loss of activity of -20°C samples (storage temperature of the stock).

DISCUSSION

The main aim of this study was to value assign a replacement international standard for antithrombin concentrate. It also provided an opportunity to re-examine the relationship between the plasma and concentrate standards.

Good agreement of potencies and low intra-laboratory variability (% GCV's) for all candidate samples were observed, indicating the participants were all able to assay antithrombin with precision and accuracy. As shown by the GCV's in tables 2 – 5 and 7 -10, the intra-laboratory (between assay) reproducibility was good. With the exception of one laboratory, the GCVs for the functional assays were all below 6%. There was no obvious correlation between performance and parameters such as methods or reagents. For the antigenic assays, with the exception of one laboratory, the GCVs were also below 6%.

When proposed candidates were assayed against the 2nd International Standard for Antithrombin Concentrate, Human, the reproducibility between laboratories was excellent, with %GCVs of 5.0, 3.2 and 3.3 for functional activity of sample B, C, and D; and 4.7, 9.9 and 10.8 for antigen measurement of sample B, C and D respectively (table 11). Variability was also low when the plasma standard was assayed against the concentrate standard, with GCVs of 8.0% and 6.8% for function and antigen respectively (table 11).

There is good continuity of unit between the plasma and concentrate standards for functional activity. For the antigen measurement there is a 7% difference between the assigned and estimated value and this may require further monitoring.

Based on the %GCV and the stability profile of all the candidates, bearing in mind that the higher GCV for the antigen measurement for samples C and D is due to higher values from one laboratory, there is little to choose between samples B, C, and D. Sample B, the recombinant product, compared well against the current IS and the other candidates, but there is currently only one recombinant product licenced and others are all plasma derived. This suggests that samples C and D may be more suitable as the next international standard. Table 12 summarises some of the physical characteristics of the 3 preparations. It is clear that the coefficient of variation of the fill (which gives an index of homogeneity of the batch) and residual moisture (which may have an effect on long term stability) are both very similar for the three candidates. However, as there are more ampoules of sample C, it is proposed therefore to recommend sample C, 06/166 to be the 3rd International Standard for Antithrombin, Concentrate, Human, with the following assigned values:

Function: 4.4 IU/ampoule

Antigen: 4.5 IU/ampoule

PARTICIPANTS RESPONSE

All participants agreed with the proposals. Two laboratories raised questions on the model fitting of the raw data analysis. This does not have any impact on the final potency assignment of the proposed replacement standard.

SSC EXPERTS RESPONSES

All nine experts who responded to the review request agreed with the proposal and recommendation.

PRODUCT SUMMARY FOR 06/166

The final ampouled material was produced as described by Campbell, 1974⁵ and the finished product summary is as follows:

Code number	06/166
Presentation	Sealed, glass 5 ml DIN ampoules
Number of ampoules available	9400
Date filled	12 October 2006
Precision of fill – CV of fill mass (% , n = 196)	0.21
Residual moisture after lyophilisation (% , n = 12)	0.12
Mean dry weight (g, n=12)	0.28
Mean oxygen content (% , n=10)	0.50
Storage conditions	-20 °C
Address of processing facility	NIBSC, Potters Bar, EN6 3QG, UK
Address of present custodian	NIBSC, Potters Bar, EN6 3QG, UK

Appendix 3 shows the proposed Instruction For Use and Material Safety Data for this preparation.

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Table 1. Functional Activity: Potency estimates (IU/ampoule) from individual assays:

Lab	Sample	Assay 1	Assay 2	Assay 3	Assay 4	Geometric Mean
1	B	3.96	4.45	4.19	.	4.20
	C	3.85	4.28	4.1	.	4.07
	D	4.19	.	4.2	.	4.19
	P	0.93	0.94	0.97	.	0.95
2	B	4.74	4.41	4.51	4.58	4.56
	C	4.82	4.49	4.4	4.97	4.66
	D	4.93	4.4	4.63	4.97	4.73
	P	0.9	0.8	0.86	0.93	0.87
3	B	4.71	4.81	4.54	4.59	4.66
	C	4.21	4.57	4.28	4.65	4.42
	D	4.48	4.69	4.3	4.72	4.54
	P	0.76	0.82	0.79	0.8	0.79
4	B	4.15	4.17	4.44	4.27	4.26
	C	4.42	4.34	4.62	4.6	4.49
	D	4.37	4.45	4.51	4.8	4.53
	P	0.74	0.77	0.77	0.79	0.77
5	B	4.36	4.1	4.02	4.38	4.21
	C	4.5	4.26	4.46	4.3	4.38
	D	3.98	4.06	4.46	4.38	4.22
	P	0.82	0.84	0.84	0.83	0.83
6	B	4.09	4.03	4.13	4.2	4.11
	C	4.42	4.38	4.26	4.48	4.38
	D	4.37	4.31	4.18	4.36	4.30
	P	0.83	0.81	0.81	0.8	0.81
7	B	.	4.6	3.23	3.53	3.74
	C
	D	4.24	5.54	.	3.5	4.35
	P	0.87	1.02	.	.	0.94
8	B	4.42	4.39	4.47	4.47	4.44
	C	4.63	4.6	4.64	4.68	4.64
	D	4.61	4.48	4.55	4.61	4.56
	P	0.83	0.8	0.81	0.84	0.82
9	B	4.17	4.42	4.55	4.43	4.39
	C	4.18	4.57	4.69	4.50	4.48
	D	4.66	4.36	4.36	.	4.46
	P	0.84	0.91	0.95	0.89	0.90
10	B	4.31	4.4	4.13	4.01	4.21
	C	4.44	4.3	4.42	4.23	4.35
	D	4.29	4.32	4.45	4.44	4.37
	P	0.75	0.8	0.78	0.75	0.77

Table 1. Continued

11	B	4.28	4.36	4.26	4.27	4.29
	C	4.39	4.59	4.41	4.32	4.43
	D	4.27	4.49	4.32	4.21	4.32
	P
12	B	4.19	4.2	4.2	4.21	4.20
	C	4.41	4.48	4.49	4.38	4.44
	D	4.34	4.38	4.34	4.32	4.34
	P	0.82	0.83	0.83	0.81	0.82
13	B	4.42	4.17	4.43	4.54	4.39
	C	4.53	4.60	4.70	4.83	4.66
	D	4.50	4.42	4.80	4.62	4.58
	P	0.85	0.79	0.87	0.82	0.83
14	B	4.48	4.39	4.35	4.69	4.48
	C	4.66	.	4.45	4.28	4.46
	D	4.22	4.24	4.29	4.2	4.24
	P	0.97	1.01	0.99	1.03	1.00
15	B	4.32	4.23	4.1	4.29	4.23
	C	4.48	4.31	4.1	4.53	4.35
	D	4.25	4.3	4.14	4.55	4.31
	P	.	0.78	0.79	0.77	0.78
16	B	<i>4.15</i>	4.27	<i>4.09</i>	<i>4.26</i>	4.19
	C	<i>4.4</i>	4.41	<i>4.25</i>	4.38	4.36
	D	<i>4.31</i>	4.38	<i>4.14</i>	4.27	4.27
	P
16a	B	<i>5.58</i>	<i>5.77</i>	<i>5.72</i>	5.71	5.69
	C	<i>4.59</i>	<i>4.28</i>	<i>4.4</i>	4.26	4.38
	D	4.56	<i>4.62</i>	<i>4.48</i>	4.48	4.53
	P	0.85	<i>0.88</i>	<i>0.84</i>	0.87	0.86
17	B	3.82	3.83	4.12	4.02	3.95
	C	4.07	3.94	4.28	4.24	4.13
	D	4.25	3.97	4.28	4.13	4.16
	P	0.85	0.87	0.85	0.9	0.87
19	B	4.24	4.4	4.44	4.7	4.44
	C	4.41	4.48	4.55	4.45	4.47
	D	4.47	4.55	4.42	4.5	4.48
	P	0.78	.	0.77	0.84	0.80
20	B	4.2	4.28	4.38	4.18	4.26
	C	4.21	4.39	4.64	4.2	4.36
	D	4.21	4.53	4.47	4.25	4.36
	P	0.75	0.82	0.82	0.79	0.79
21	B	<i>4.26</i>	<i>4.21</i>	4.39	<i>4.36</i>	4.30
	C	<i>4.51</i>	<i>4.14</i>	4.16	<i>4.52</i>	4.33
	D	<i>4.56</i>	<i>4.39</i>	4.39	<i>4.46</i>	4.45
	P	<i>0.81</i>	<i>0.77</i>	0.78	<i>0.82</i>	0.79

Estimates in bold and Italic are not valid at 1% level of inference, but visual inspection of the dose-response curve was satisfactory.

Table 2. Functional Activity: potency estimates in IU/ampoule for sample B.

Lab	Geometric Mean Potency ¹	% GCV	Geometric Mean Potency ²	% GCV
01	4.20*	6.0	4.34	12.6
02	4.56	3.1	4.62	3.8
03	4.66	2.6	4.70	2.5
04	4.26	3.1	4.23	2.8
05	4.21	4.4	4.26	4.3
06	4.11	1.7	4.08	2.8
07	3.74*	20.2	3.82	20.3
08	4.44	0.9	4.44	1.6
09	4.39	3.7	4.46	2.6
10	4.21	4.3	4.25	4.2
11	4.29	1.1	4.29	1.2
12	4.20	0.2	4.20	0.2
13	4.12	3.7	4.18	4.7
14	4.48	3.4	4.47	3.4
15	4.23	2.3	4.24	2.3
16	4.19	2.1	4.15	3.5
16a	5.69	1.4	5.82	1.6
17	3.95	3.8	3.93	3.5
19	4.44	4.4	4.43	3.4
20	4.26	2.1	4.51	2.8
21	4.30	2.0	4.86	2.3
Geometric Mean	4.32		4.38	
95% C.I.	(4.16-4.48)		(4.21-4.55)	
%GCV	8.3		9.0	
Excluding lab 16a:	4.26		4.32	
	(4.16-4.36)		(4.2-4.44)	
	5.0		5.9	

¹Calculated relative to sample A at NIBSC.²Calculated from estimates reported by laboratory.

*Calculated from 3 assays only.

Table 3. Functional Activity: potency estimates in IU/ampoule for sample C

Lab	Geometric Mean Potency ¹	% GCV	Geometric Mean Potency ²	% GCV
01	4.07*	5.5	4.06	2.4
02	4.66	5.9	4.80	5.2
03	4.42	5.0	4.42	5.1
04	4.49	3.1	4.50	2.6
05	4.38	2.7	4.40	2.5
06	4.38	2.2	4.38	1.4
07	.	.	4.19	8.8
08	4.64	0.7	4.64	1.8
09	4.48	5.1	4.53	3.4
10	4.35	2.3	4.37	4.8
11	4.43	2.6	4.43	2.6
12	4.44	1.2	4.44	1.4
13	4.66	2.8	4.41	2.5
14	4.46*	4.4	4.42	4.1
15	4.35	4.6	4.33	4.2
16	4.36	1.7	4.28	2.4
16a	4.38	3.5	4.35	3.8
17	4.13	3.9	4.09	3.9
19	4.47	1.3	4.44	1.5
20	4.36	4.8	4.64	1.9
21	4.33	5.0	4.92	3.3
Geometric Mean 95% C.I. %GCV	4.40 (4.34-4.46) 3.2		4.43 (4.34-4.52) 4.7	

¹ Calculated relative to sample A at NIBSC.

² Calculated from estimates reported by laboratory.

*Calculated from 3 assays only.

Table 4. Functional Activity: potency estimates in IU/ampoule for sample D

Lab	Geometric Mean Potency ¹	% GCV	Geometric Mean Potency ²	% GCV
01	4.19	.	4.67	14.5
02	4.73	5.9	4.84	5.7
03	4.54	4.5	4.57	3.3
04	4.53	4.2	4.54	4.1
05	4.22	5.7	4.26	5.2
06	4.30	2.1	4.34	3.7
07	4.35*	25.9	4.02	22.7
08	4.56	1.4	4.57	2.4
09	4.46*	3.9	4.48	5.1
10	4.37	1.9	4.40	4.2
11	4.32	2.8	4.32	2.7
12	4.34	0.6	4.35	0.8
13	4.58	3.6	4.38	1.4
14	4.24	0.9	4.23	1.0
15	4.31	4.1	4.30	3.9
16	4.27	2.4	4.16	2.2
16a	4.53	1.5	4.52	1.7
17	4.16	3.5	4.14	3.7
19	4.48	1.2	4.46	1.8
20	4.36	3.7	4.64	4.3
21	4.45	1.8	4.99	1.4
Geometric Mean	4.38		4.43	
95% C.I.	(4.32-4.45)		(4.33-4.54)	
%GCV	3.3		5.4	

¹ Calculated relative to sample A at NIBSC.

² Calculated from estimates reported by laboratory.

*Calculated from 3 assays only.

Table 5. Functional Activity: potency estimates in IU/ampoule for sample P

Lab	Geometric Mean Potency ¹	% GCV	Geometric Mean Potency ²	% GCV
01	0.95*	2.2	0.99	18.0
02	0.87	6.7	0.87	6.1
03	0.79	3.2	0.79	2.9
04	0.77	2.7	0.76	1.7
05	0.83	1.2	0.84	1.1
06	0.81	1.6	0.81	1.9
07	0.94	.	0.78	18.5
08	0.82	2.3	0.82	2.0
09	0.90	5.3	0.89	4.0
10	0.77	3.2	0.76	3.3
11
12	0.82	1.2	0.82	1.3
13	0.83	4.3	0.78	3.5
14	1.00	2.6	1.00	3.0
15	0.78*	1.3	0.79	1.9
16	.	.	0.86	3.5
16a	0.86	2.1	0.78	4.2
17	0.87	2.7	0.73	3.0
19	0.80*	4.8	0.76	3.3
20	0.79	4.3	0.84	5.2
21	0.79	3.0	0.91	1.4
Geometric Mean	0.84		0.83	
95% C.I.	(0.81-0.87)		(0.79-0.86)	
%GCV	8.0		8.9	

¹ Calculated relative to sample A at NIBSC.

² Calculated from estimates reported by laboratory.

*Calculated from 3 assays only.

Figure 1. Histogram of results of functional activity assays for sample B:

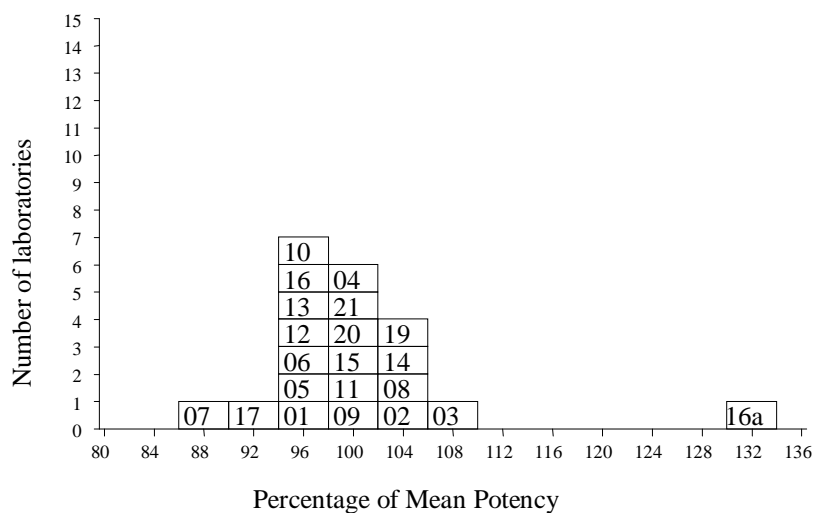


Figure 2. Histogram of results of functional activity assays for sample C:

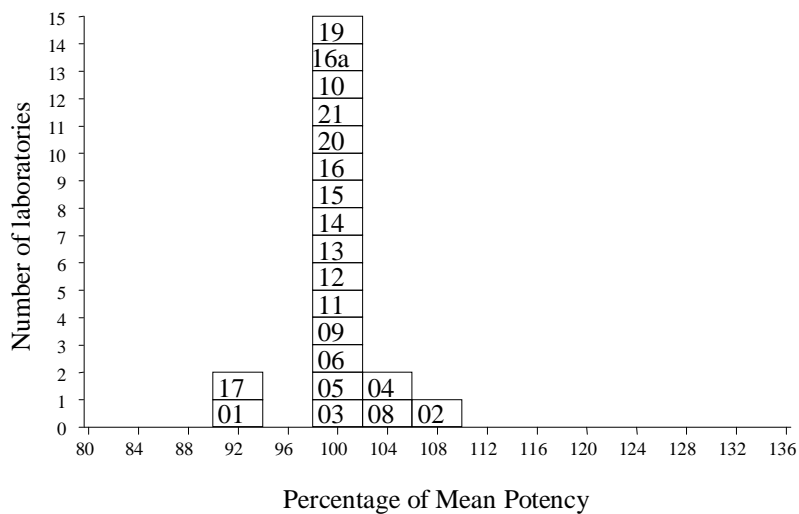


Figure 3. Histogram of results of functional activity assays for sample D:

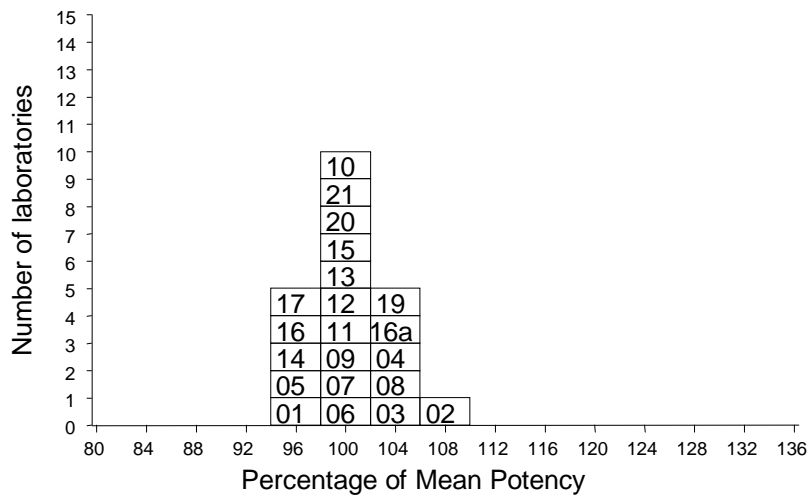


Figure 4. Histogram of results of functional activity assays for sample P:

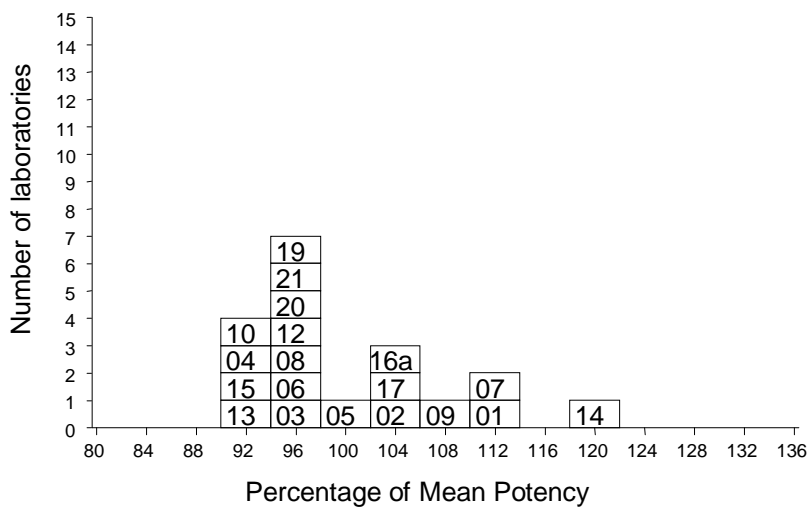


Table 6. Antigen: potency estimates (IU/ampoule) from individual assays.

Lab	Sample	Assay 1	Assay 2	Assay 3	Assay 4	Geometric Mean
02	B	4.00	4.00	4.10	4.10	4.05
	C	4.00	4.10	4.10	4.20	4.10
	D	4.00	3.90	4.00	4.00	3.97
	P	0.53	0.46	0.56	0.52	0.52
06	B	3.93	4.08	4.26	4.59	4.21
	C	4.93	5.61	5.85	5.92	5.56
	D	5.20	5.35	5.63	5.93	5.52
	P	1.07	1.21	1.25	1.26	1.19
12	B	4.50	4.48	4.42	4.48	4.47
	C	4.30	4.28	4.17	4.31	4.26
	D	4.31	4.28	4.17	4.26	4.25
	P	0.78	0.79	0.76	0.79	0.78
13	B	4.15	3.92	4.17	4.26	4.12
	C	4.26	4.32	4.42	4.54	4.38
	D	4.23	4.15	4.51	4.34	4.31
	P	0.79	0.74	0.82	0.77	0.78
14	B	3.77	4.32	4.26	4.84	4.28
	C	3.86	6.00	4.70	4.92	4.81
	D	4.13	6.39	4.68	4.62	4.89
	P	0.76	0.88	0.86	0.75	0.81
14a	B	4.73	4.72	4.96	3.57	4.46
	C	3.47	4.75	6.81	3.84	4.56
	D	3.54	4.84	4.98	3.37	4.12
	P	0.56	0.89	1.01	0.64	0.75
16	B	4.10	4.07	4.07	4.04	4.07
	C	4.09	3.97	3.99	3.95	4.00
	D	4.10	4.01	3.91	3.92	3.98
	P
18	B	4.71	4.57	4.84	4.59	4.68
	C	4.37	4.82	4.40	4.20	4.44
	D	4.03	4.46	4.07	4.06	4.15
	P	0.68	0.87	0.74	0.75	0.76
19	B	4.33	4.32	4.42	4.64	4.43
	C	4.43	4.47	4.52	4.36	4.44
	D	4.49	4.56	4.40	4.39	4.46
	P	0.79	.	0.74	0.76	0.76
21	B	4.49	4.57	4.38	4.47	4.48
	C	3.99	4.37	4.45	4.23	4.26
	D	4.17	4.33	4.30	4.15	4.24
	P	0.90	0.95	0.94	0.92	0.93

Lab 16: sample P was non-parallel in all assays.

Lab 19, assay 2: no results provided for sample P.

Table 7. Antigen: potency estimates (IU/ampoule) for sample B

Lab	Geometric Mean Potency ¹	% GCV	Geometric Mean Potency ²	% GCV
02	4.05*	1.4*	4.05	1.4
06	4.21	6.9	4.21	6.9
12	4.47	0.8	4.46	1.0
13	4.41	2.5	4.41	3.1
14	4.28	10.8	4.28	10.8
14a	4.46	16.2	4.46	16.2
16	4.07	0.6	4.18	1.0
18	4.68	2.7	4.71	5.7
19	4.43*	3.4*	4.43	3.4
21	4.48	1.8	4.44	4.5
Geometric Mean 95% C.I. %GCV	4.35 (4.21-4.49) 4.7		4.36 (4.23-4.49) 4.4	

¹ Calculated relative to sample A at NIBSC.

² Calculated from estimates reported by laboratory.

* Copied from labs reported results as data unsuitable for parallel line analysis.

Table 8. Antigen: potency estimates (IU/ampoule) for sample C

Lab	Geometric Mean Potency ¹	% GCV	Geometric Mean Potency ²	% GCV
02	4.10*	2.0*	4.10	2.0
06	5.56	8.7	5.56	8.7
12	4.26	1.5	4.25	1.9
13	4.29	2.5	4.33	3.5
14	4.81	19.8	4.81	19.8
14a	4.56	34.7	4.56	34.7
16	4.00	1.6	4.17	1.7
18	4.44	6.0	4.43	6.0
19	4.44*	1.5*	4.44	1.5
21	4.26	4.9	4.24	2.0
Geometric Mean 95% C.I. %GCV	4.45 (4.16-4.76) 9.9		4.47 (4.20-4.77) 9.4	

¹ Calculated relative to sample A at NIBSC.

² Calculated from estimates reported by laboratory.

* Copied from labs reported results as data unsuitable for parallel line analysis.

Table 9. Antigen: potency estimates (IU/ampoule) for sample D

Lab	Geometric Mean Potency ¹	% GCV	Geometric Mean Potency ²	% GCV
02	3.97*	1.3*	3.97	1.3
06	5.52	6.0	5.52	6.0
12	4.25	1.4	4.24	1.5
13	4.17	2.5	4.21	1.1
14	4.89	20.6	4.89	20.6
14a	4.12	22.7	4.12	22.7
16	3.98	2.2	4.04	4.2
18	4.15	4.9	4.23	7.7
19	4.46*	1.8*	4.46	1.8
21	4.24	2.2	4.23	3.5
Geometric Mean 95% C.I. %GCV	4.35 (4.05-4.69) 10.8		4.37 (4.07-4.70) 10.6	

¹ Calculated relative to sample A at NIBSC.

² Calculated from estimates reported by laboratory.

* Copied from labs reported results as data unsuitable for parallel line analysis.

Table 10. Antigen: potency estimates (IU/ampoule) for sample P.

Lab	Geometric Mean Potency ¹	% GCV	Geometric Mean Potency ²	% GCV
02
06	1.19	7.9	1.19	7.9
12	0.78	1.8	0.78	2.5
13	0.78	2.9	0.77	4.0
14	0.81	8.6	0.81	8.6
14a	0.75	31.8	0.75	31.8
16	.	.	0.54	1.8
18	0.76	10.8	0.78	14.3
19	0.76*	3.3*	0.76	3.3
21	0.93	2.4	0.91	2.1
Geometric Mean	0.84		0.84	
95% C.I.	(0.73-0.95)		(0.73-0.95)	
%GCV	17.2		17.0	
Excluding lab 06:	0.79		0.79	
	(0.75-0.84)		(0.74-0.85)	
	6.8		7.5	

¹ Calculated relative to sample A at NIBSC.

² Calculated from estimates reported by laboratory.

* Copied from labs reported results as data unsuitable for parallel line analysis.

Lab 2 suggested that due to the nature of sample P it was unsuitable for their procedure and that the results were invalid.

Lab 16 is not included in the geometric mean of reported results for comparability.

Figure 5. Histogram of results of antigen assays for sample B:

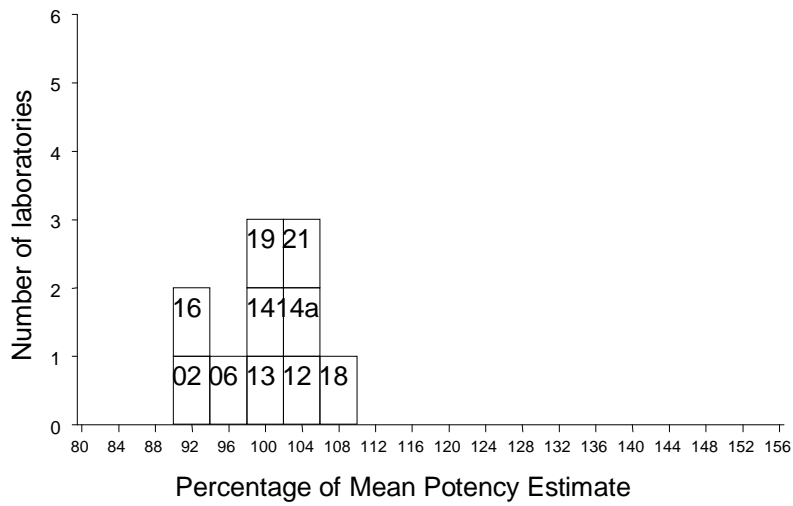


Figure 6. Histogram of results of antigen assays for sample C:

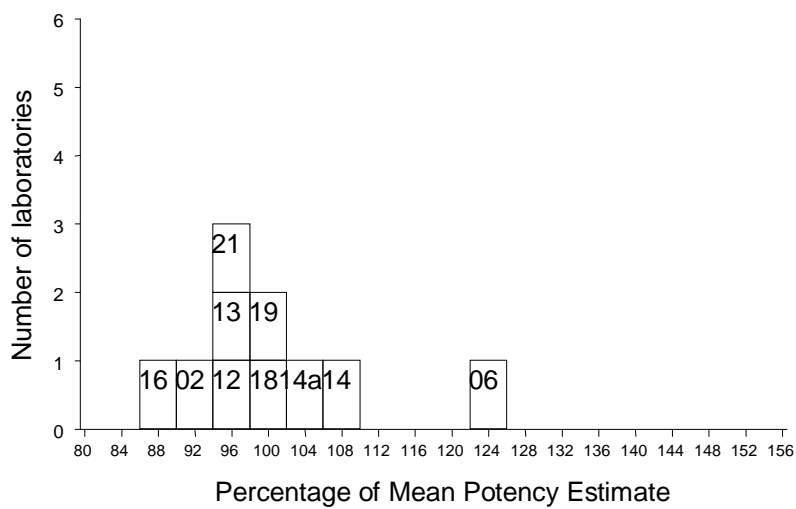


Figure 7. Histogram of results of antigen assays for sample D:

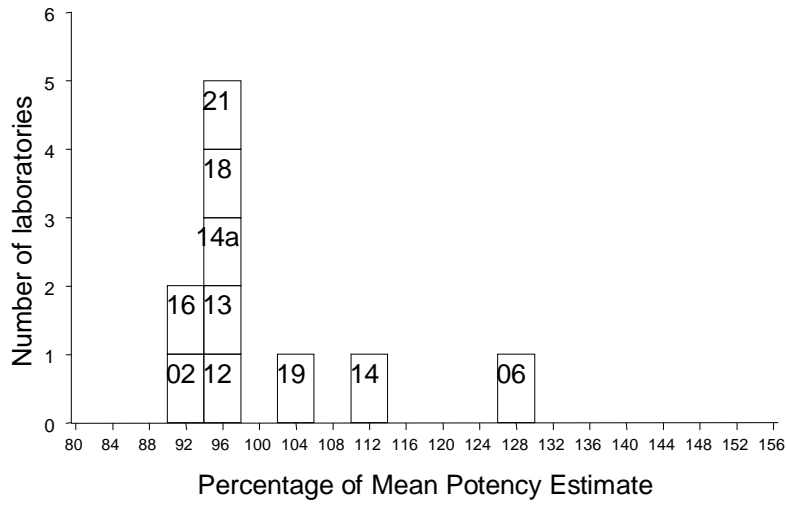


Figure 8. Histogram of results of antigen assays for sample P:

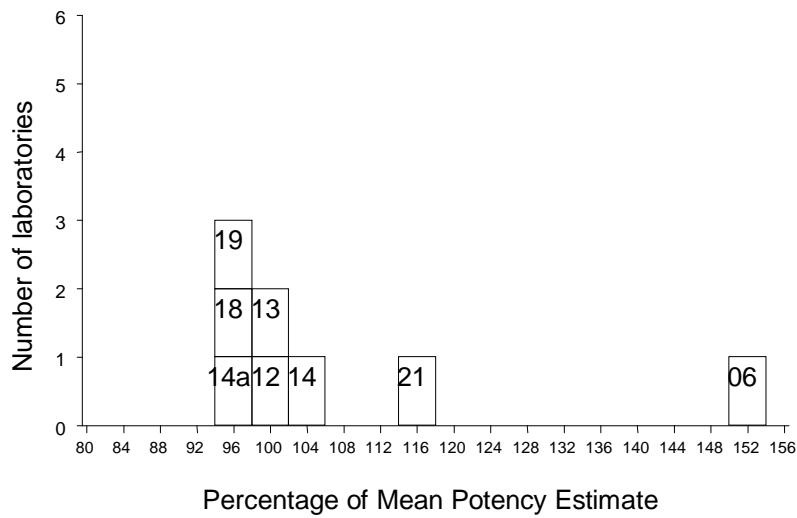


Table 11. Summary of potency estimates relative to Sample A, the 2nd IS for Antithrombin, Concentrate

Sample		Function	Antigen
B	Potency IU/amp	4.26	4.35
	GCV %	5.0	4.7
C	Potency IU/amp	4.40	4.45
	GCV %	3.2	9.9
D	Potency IU/amp	4.38	4.35
	GCV %	3.3	10.8
P	Potency IU/amp	0.84	0.79
	GCV %	8.0	6.8

Table 12. Summary of some physical characteristics of samples B, C and D

Sample	Code	No of ampoules available	Residual Moisture %	Coefficient variation of the fill %
B	06/160	8000	0.18	0.11
C	06/166	9400	0.13	0.21
D	06/168	5500	0.11	0.16

Appendix 1 List of participants

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Appendix 2 Methods used by the participants

Lab	Function – Heparin co-factor chromogenic		Antigen Measurement
	Thrombin inhibition	FXa Inhibition	
1	✓		NT
2	✓		Nephelometry
3	✓		NT
4		✓	NT
5	✓		NT
6	✓		Laurells
7	✓		NT
8	✓		NT
9	✓		NT
10		✓	NT
11	✓		NT
12	✓		Nephelometry
13	✓		Elisa
14	✓		14 Laurells, and 14a Elisa
15	✓		NT
16	✓	16a ✓	Nephelometry
17	✓		NT
18			Elisa
19		✓	Nephelometry
20	✓		NT
21	✓		Immunoturbidimetric

NT = not tested

Appendix 3: Proposed Instruction For Use and Material Safety Data

The 3rd International Standard for Antithrombin, Concentrate, Human 06/166 (established 2007) Instructions for Use (Version 1 July 2007)

1. INTRODUCTION

The 3rd International Standard for Antithrombin, Concentrate, Human, consists of ampoules, coded 06/166, containing aliquots of a freeze-dried concentrate prepared from human plasma. This preparation was established as the 3rd International Standard for Antithrombin, Concentrate, Human, by the Expert Committee on Biological Standardisation of the World Health Organisation in 2007.

The ECBS report is available from the WHO (www.who.int/biologicals). Document number: WHO/BS/07.....

2. UNITAGE

The standard was value assigned in an international collaborative study involving 21 laboratories from 12 countries against the 2nd International Standard for Antithrombin, Concentrate, Human, 96/520. All the functional assays performed were based on the heparin co-factor chromogenic method (17 labs used thrombin inhibition, 4 labs used factor Xa inhibition). The antigenic assays were carried out by nephelometry (4 labs), Laurells (2 labs), 3 ELISA (3 labs) and immunoturbidimetry (1 lab). The assigned potencies are as follows:

Functional : 4.4 IU/ampoule
Antigenic: 4.5 IU/ampoule

Uncertainty: the assigned unitage does not carry an uncertainty associated with its calibration. The uncertainty may therefore be considered to be the variance of the ampoule content and was determined to be +/- 0.21 %.

3. CONTENTS

Thirty-four vials (1500 IU/vial) of plasma derived human antithrombin concentrate were each reconstituted with 30 ml of sterile distilled water. Following the dilution of the pooled material with approximately 9 litres of 0.05M Tris, 0.15M NaCl, pH 7.4 containing 2 mg/ml trehalose and 10 mg/ml human albumin. the solution was distributed at 4°C into 10,000 ampoules, coded 06/166. The mean weight of liquid content of 196 check weight ampoules was 1.0055g, with limits of 1.0000 - 1.0095 g (coefficient of variation 0.21%). The contents of the ampoules were then freeze-dried under the conditions normally used for international biological standards¹.

4. CAUTION

THIS PREPARATION IS NOT FOR ADMINISTRATION TO HUMANS.

The preparation contains material of human origin, which has been tested and found negative for HBsAg, HIV antibody, HCV antibody and HCV RNA by PCR.

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 probably will include the wearing of protective gloves and avoiding the generation of aerosols. Care should be exercised in opening ampoules or vials, to avoid cuts.

5. DIRECTIONS FOR OPENING THE DIN AMPOULE

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.

6. USE OF AMPOULED MATERIAL

Unopened ampoules should be stored in the dark at or below -20°C .

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. Stand for 10 minutes at room temperature to allow complete dissolution of the material before use. The reconstituted Standard should be used as soon as possible.

7. STABILITY

It is the policy of WHO not to assign an expiry date to their international reference materials. They remain valid with the assigned potency and status until withdrawn or amended.

Reference materials are held at NIBSC within assured, temperature-controlled storage facilities and should be stored on receipt as indicated on the label. Once reconstituted, diluted or aliquoted, users should determine the stability of the material according to their own method of preparation, storage and use.

NIBSC follows the policy of WHO with respect to its reference materials. A preliminary accelerated degradation study, involving the potency estimation of ampoules stored at elevated temperatures (4, 20, 37, 45 °C) relative to ampoules stored at -150°C was carried out in one laboratory (NIBSC), using the heparin co-factor chromogenic thrombin based assay. The observed relative loss of potency was analysed using the Arrhenius equation in order to provide a prediction of loss per year for ampoules stored at various temperatures. Estimates of % predicted loss of activity per year based on activities assessed at 3 different time points over a period of 9

months storage showed that there was no predicted loss of activity for samples stored at -20°C, the storage temperature of the proposed IS, thus indicating that the preparation is exceedingly stable and suitable for long term use as an International Standard. The accelerated degradation study and real time monitoring will continue for the lifetime of the standard.

Users who have data supporting any deterioration in the characteristics of any reference preparation are encouraged to contact NIBSC.

8. CITATION

In any circumstance where the recipient publishes a reference to NIBSC materials, it is important that the title of the preparation and any NIBSC code number, and the name and address of NIBSC are cited correctly.

9. 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 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 dependent 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.

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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.

10. References

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

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