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A COLLABORATIVE STUDY FOR VALUE ASSIGNMENT OF THE 3RD INTERNATIONAL STANDARD FOR PROTEIN S, PLASMA

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NOTE:
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 **2 October 2023** and should be addressed to the World Health Organization, 1211 Geneva 27, Switzerland, attention: Technical Standards and Specifications (TSS). Comments may also be submitted electronically to the Responsible Officer: **Dr Ivana Knezevic** at email: knezevici@who.int.

Summary

Twenty-one laboratories from 8 countries agreed to participate in a collaborative study to establish a replacement for the 2nd International Standard for Protein S, Plasma (03/228). Locally sourced normal pooled plasmas were also included in the study to assess the relationship between the International Unit (IU) and the normal plasma unit. The laboratories were able to perform the functional and antigenic assays with reasonable precision; when assayed against the 2nd International Standard (IS), intra-laboratory geometric coefficients of variation (GCV) ranged from 0.6 to 13.5%, 0.2 to 16.8% and 1.3 to 14.1% for the functional, free and total antigen assays respectively, with the majority being below 5%. For the functional and free antigen assays, good agreement was observed between laboratories, evidenced by low inter-laboratory GCV for the candidate material, and the included common plasma sample (4.5% and 4.3% functional and 5.2% and 5.6% free). For the total antigen measurement, the inter-laboratory variation was higher for the common sample, 10.5% but good for the candidate at 3.2%. Complete analysis against the normal plasma unit was not possible due to some laboratories not including their own pool of plasma. However, relative to the International Standard the value for the overall potency of the pools was 1.0 IU/ml for all analytes confirming the original definition of the IU and highlighting the long-term stability of the current IS and suitability to calibrate the replacement.

It is recommended that the candidate material, sample B (NIBSC code, 22/202) be considered as the 3rd International Standard for Protein S, Plasma, with assigned potencies:

functional activity:	**0.71 IU/ampoule.**
free antigen:	**0.83 IU/ampoule.**
total antigen:	**0.88 IU/ampoule**.

Introduction

Protein S is a vitamin K co-factor for protein C, which is an inhibitor within the coagulation cascade. A lack of protein S can lead to thrombophilia; therefore, a protein S International Standard was required to enable diagnostic measurement of protein S levels. Protein S deficiency can exist as three types – type I, II & III – the type of which can be determined diagnostically by the three analytes – functional, free antigen & total antigen – associated with the protein S. In addition to diagnostic use, the protein S reference material is also used to measure protein S levels in some therapeutic products, virus inactivated fresh frozen plasma and some prothrombin complex concentrates.

The 2nd International Standard (IS) for Protein S, Plasma, 03/228 was established in 2006 – WHO/BS/06.2046. Due to the depletion of stock, a replacement is required. In the present study, a batch of freeze-dried normal pooled plasma was evaluated, and value assigned against the 2nd International Standard for Protein S, Plasma, 03/228, with a view to establishing this candidate material as the 3rd International Standard for Protein S, Plasma. Participants were also requested to include locally collected normal plasma pools to allow for assessment of the relationship between the International Unit (IU) and the normal plasma pool unit of protein S.

Participants

A list of the twenty-one participants is given in the 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 nineteen laboratories from 8 different countries including 4 clinical laboratories, 5 therapeutic manufacturer, 2 regulatory control laboratories, and 8 diagnostic manufacturers returned results. The two laboratories who were not able to return data were 5 and 21.

The Candidate, code 22/202

Thirty donations of platelet poor normal plasma from the UK's National Blood and Transfusion Service (NBTS), collected in CPD-adenine and double spun prior to freezing at -70ºC. On the day of the fill the plasma was thawed at 37ºC, pooled and buffered with 0.05 M HEPES and 1% glycine, prior to being distributed into siliconized glass ampoules, filled and freeze-dried according to guidelines for production of international biological standards [1] [2]. Each individual plasma donation had been tested and found negative for anti-HIV 1/2, HBsAg and anti-Hepatitis C prior to being supplied by NBTS. This candidate was coded as sample B in this study. The product characteristics are shown in Appendix 2, and it should be noted that the fill volume per ampoule was 1.1 g to correct for the dilution effect of the buffering material.

Samples

The following coded samples were sent to the participants:

A – the 2nd International Standard (03/228) for protein S, plasma.
Functional – 0.77 IU/ampoule
Free antigen – 0.81 IU/ampoule

Total antigen – 0.83 IU/ampoule

B – proposed 3rd International Standard (22/202) for protein S, plasma.
Functional – approximately 0.7 IU/ampoule
Free antigen – approximately 0.8 IU/ampoule
Total antigen – approximately 0.8 IU/ampoule

C – common plasma sample
All activities approximately 0.6 IU/ampoule

In addition, the participants were requested to collect normal pooled plasma which was coded P in the study:

P - Local fresh and frozen normal pooled plasma were included and requested to be collected according to the protocol provided (Appendix 3) where feasible. Only one laboratory used fresh and frozen plasma, with other laboratories who included local pooled plasma using either a locally collected frozen pooled plasma or commercially sourced frozen normal pooled plasma with one lab including pools from two different sources. In total 13 laboratories included a normal pooled plasma.

Assay Methods

Each participant was requested to perform their routine in-house analytical method(s) for protein S, and where multiple analytes were measured to perform this on the same set of supplied samples.

All the functional assays performed were clotting based with several different commercial methods used. Ten laboratories returned results, with several laboratories returning data from several methods, totalling 16 sets of assay data.

Sixteen laboratories performed free antigen assays, with several laboratories carrying out different types of free antigen method. In total, 24 sets of results were returned: 6 sets were ELISA based and the remaining 18 were immunoturbidimetric techniques.

Eight laboratories performed total antigen assay, with two laboratories returned results from two different methods. In total ten sets of results were returned with all methods used being ELISA based.

A list of methods performed by the participants is given in Appendix 4.

Study Design

Participants were requested to perform four independent assays for each type of method. Where feasible, participants were requested to include a normal pooled plasma (P). It was requested that each fresh pool was tested in the study on the day of collection and that a frozen sample of the same pool should be used in the study on a separate day, only one laboratory performed this with all other laboratories who included a pooled plasma using frozen batches. The participants were to assay concurrently a series of at least three dilutions of each of the

three/four study samples. The assay order of the materials was varied to give an overall balanced order of testing with duplicate measurements on the same dilutions. Participants were requested to return raw assay data, along with their own estimates for the protein S potency of materials B, C and P using A as the standard.

Statistical Analysis

Assay Data

Functional activity assays were performed by 10 laboratories. In total, 62 functional assays were considered for analysis where each laboratory performed 4 assays apart from: lab 3 which performed three types of assay, two for which only three set of data were returned; lab 13 which performed two types of assay; and lab 14 which performed four types of assay.

Free antigen assays were performed by 16 laboratories. In total, 96 free antigen assays were analysed as each laboratory performed 4 assays apart from lab 6, 13 and 18 which performed two types of free antigen assay and lab 14 which performed six types of free antigen assay.

Total antigen assays were performed by 8 laboratories. In total, 44 free antigen assays were analysed as each laboratory performed 4 assays apart from lab 14 and 15 which performed two types of total antigen assay.

Analysis Methods

An independent statistical analysis of raw data was performed at MHRA. Relative potency estimates were calculated by fitting a parallel-line or slope-ratio model (3). All data were plotted, and assay validity was assessed both visually and by analysis of variance. All mean potencies given in this report are unweighted geometric mean (GM) 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 estimates). Grubbs' Test (4) was applied to the log transformed laboratory mean estimates in order to detect any significant outliers ($p<0.05$). Any comparison of groups has been performed using a Students' T-Test with log transformed laboratory means.

Functional Assay

A slope-ratio model (log-transformed response for laboratories 3a, 3b, 3c, 8, 14a, 14b, 14c, 19; untransformed for laboratories 4, 12, 18) or parallel-line model (log-transformed response for laboratories 10, 13a, 13b, 20 and untransformed for laboratory 14d) comparing assay responses was used to estimate the potency of samples relative to sample A.

Free Antigen Assays

A parallel-line model with log-transformation of response was used for all laboratories to compare assay responses to estimate the potency of samples relative to sample A.

Total Antigen Assays

A parallel line model (untransformed response for laboratories 3, 15a, 17 and log-transformed for laboratories 4, 11, 14a, 14b,18) was used for all apart from 15b where a sigmodal curves model was used. Analysis of data from laboratory 16 was found not to fit to any model due to poor regression and has been excluded from the study.

Results

Assay Validity

Almost all functional assays were found to fit to the models showing no significant ($p<0.01$) deviations from the fitted model. The only exceptions were assay 2 from laboratory 8 which was found to have high variability due to poor duplicate responses and sample P in assay 3 from laboratory 12 where the sample deviated from the model – values were excluded from further analysis.

Apart from one sample in one assay, all free antigen assays were found to fit to the models showing no significant ($p<0.01$) deviations from the fitted model. The exception was sample C in assay 1 from laboratory 7 which was found to be non-linear and was excluded from analysis.

Apart from one assay, all total antigen assays were found to fit to the models showing no significant ($p<0.01$) deviations from the fitted model. The exception was assay 2 from laboratory 11 which was found to have high variability due to poor duplicate responses and was excluded from analysis.

Intra- and Inter-Laboratory Variability

Functional Activity: Individual assay potency estimates and intra-laboratory (within laboratory) variability, expressed as GCVs, for samples B, C and P are listed in Tables 1a – 1c. The majority of intra-laboratory GCV values were below 10%, with 59% of GCVs being less than 5% (Table 7). Within each laboratory, all samples assayed well against sample A, the 2nd IS, with higher variation seen with the plasma pool. A higher variability in the GCV (several laboratories had GCVs greater than 10%) for the plasma pools used in some laboratories was not unexpected given the request to use different pools in the study.

The overall inter-laboratory GCVs were 4.5%, 4.3% and 5.6% for samples B, C and P with a slightly higher GCV for P, the local normal pooled plasmas.

Free Antigen Activity: Details of the calculated values for each individual assay and intra-laboratory variability for samples B, C and P relative to sample A can be found in Table 2a – 2c. The majority of intra-laboratory GCV values were below 10%, with 66% of GCVs being less than 5% (Table 7). Within each laboratory, all samples assayed well against sample A, the 2nd IS, with higher variation seen with the plasma pool. A higher variability in the GCV (several laboratories had GCVs greater than 10%) for the plasma pools used in some laboratories was not unexpected given the request to use different pools in the study.

The overall inter-laboratory GCVs were 5.2% and 5.6% for samples B and C with a slightly higher GCV for P, 6.4%, the local normal pooled plasmas.

Total Antigen Activity: Details of the calculated values for each individual assay and intra-laboratory variability for samples B, C and P relative to sample A can be found in Table 3a – 3c. Less than half (33%) the intra-laboratory GCV values were below 5%, which indicated that the total antigen assay was variable (Table 7). Whilst within each laboratory, the samples assayed well against sample A, the 2nd IS, intra-laboratory GCVs higher than 5% were observed across all samples.

The overall inter-laboratory GCVs were 3.2%, 10.5% and 3.7% for samples B, C and P respectively.

Potency Estimates relative to Sample A, the 2nd IS and recalculated against sample B.

Functional Activity

Details of the calculated values for each individual functional assay and the geometric mean potencies for samples B, C and P relative to sample A, the 2nd IS, can be found in Tables 1a to 1c. These data are also shown in scatter plot form in figure 1. No outliers were found using Grubbs' Test. The intra-laboratory geomeans were 0.71 IU/ampoule for sample B, 0.62 IU/ampoule for sample C and 1.00 IU/ml for the pooled plasmas.

A summary of the laboratory geomean for sample C relative to sample A, the 2nd IS and recalculated relative to sample B, the candidate material with an assigned value of 0.71 IU/ml is shown in table 4. Reanalysis of sample C and the local pool relative to sample B gave activity estimates that were not significantly different (p=0.624, 0.923) when the samples were estimated against sample A – for sample C the geomeans were both 0.62 IU/ampoule, and for the pooled plasma the values were both 0.99 IU/ml.

No subgroup analysis, to compare different methods, was feasible due to insufficient numbers for the different commercial methods that were used in the measurement of functional protein S.

Free Antigen Activity

Details of the calculated values for each individual free antigen assay and the geometric mean potencies for samples B, C and P relative to sample A, the 2nd IS, can be found in Tables 2a to 2c. These data are also shown in scatter plot form in figure 2. No outliers were found using Grubbs' Test. The intra-laboratory geomeans were 0.83 IU/ampoule for sample B, 0.73 IU/ampoule for sample C and 1.03 IU/ml for the pooled plasmas.

A summary of the laboratory geomean for sample C relative to sample A, the 2nd IS and recalculated relative to sample B, the candidate material with an assigned value of 0.83 IU/ml is shown in table 5. Reanalysis of sample C and the local pool relative to sample B gave activity estimates that were not significantly different (p=0.663, 0.515) when the samples were estimated against sample A – for sample C the geomeans were both 0.73 IU/ampoule, and for the pooled plasma the values were both 1.03 and 1.04 IU/ml respectively (against sample A and against sample B).

No subgroup analysis, to compare different methods, was feasible due to insufficient numbers for the different commercial methods that were used in the measurement of free protein S antigen.

Total Antigen Activity

Details of the calculated values for each individual free antigen assay and the geometric mean potencies for samples B, C and P relative to sample A, the 2nd IS, can be found in Tables 3a to 3c. These data are also shown in scatter plot form in figure 3. No outliers were found using

Grubbs' Test. The intra-laboratory geomeans were 0.88 IU/ampoule for sample B, 0.84 IU/ampoule for sample C and 1.02 IU/ml for the pooled plasmas.

A summary of the laboratory geomean for sample C relative to sample A, the 2nd IS and recalculated relative to sample B, the candidate material with an assigned value of 0.88 IU/ml is shown in table 6. Reanalysis of sample C and the local pool relative to sample B gave activity estimates that were not significantly different (p=0.658, 0.710) when the samples were estimated against sample A – for sample C the geomeans were 0.84 and 0.83 IU/ampoule respectively (against sample A and against sample B) and for the pooled plasma the values were both 1.02 IU/ml.

The low number of laboratories who carried out total protein S antigen mean that any method comparison is not feasible.

Stability Studies

Accelerated degradation study

Preliminary accelerated degradation (5) study of the proposed IS, 22/202, monitored using a functional assay, consider the most appropriate analyte for measuring degradation, after 6 months storage (2 time points: 3 and 6 months) at temperatures of -70, -20, +4, +20, +37 and +45°C, gave a predicted loss of 0.013% per year when stored at -20°C (upper 95% confidence limit = 0.133% per year), see appendix 5 for predictive stability. Further accelerated degradation study at elevated temperature will be carried out to monitor the stability of the replacement standard.
In addition, real time monitoring of -20°C ampoules (storage temperature of stock) against -70°C samples will be performed throughout the lifespan of the material.

On-bench stability

It is recommended that upon reconstitution, the ampoule content should be transferred to a plastic tube and stored on melting ice. Although assays should be performed as soon as possible after reconstitution, results from functional and free antigen assays carried out at MHRA indicated that the activity is stable for up to 4 hours if the reconstituted sample is kept on melting ice (% residual activity after 4 hours on melting ice relative to freshly reconstituted material: 98% functional and 94% free antigen).

The use of frozen and thawed aliquots of the proposed 3rd IS is not recommended.

Discussion

The aim of this study was to value assign a replacement International Standard for Protein S Plasma.

As shown in Tables 1 to 3, there was generally good agreement of potencies against the 2nd IS with low intra-laboratory % GCV for the provided samples (B & C), indicating that the participants were able to measure protein S with precision and accuracy. The GCVs for the functional assays ranged from 0.3 to 13.5% with 23 out of 32 laboratories' mean potency estimates achieving GCVs of less than 5% (Table 7). The intra-laboratory % GCV for the

plasma pool was higher than for the supplied samples, 2.1 to 16.9%, with the majority (57%) of GCVs above 5%. The inter-laboratory GCVs (Table 8) were B – 4.5%, C – 4.3% and the plasma pools at 5.6%.

For the free antigen assays, the GCVs ranged from 0.2% to 16.8% with 34 out of the 48 potency estimates having GCVs of less than 5% (Table 7). The intra-laboratory % GCV for the plasma pool was higher than for the supplied samples, ranging from 0.7 to 11.4%, with the of % GCVs above 5%. The inter-laboratory GCV for free antigen were B – 5.2%, C – 5.6% and the plasma pools at 6.4%.

For the total antigen assays, the GCVs were slightly higher, ranging from 1.3% to 14.1% with most potencies 11 out of the 18 potency estimates having GCVs of greater than 5% (Table 7). The inter-laboratory GCV for free antigen were B – 3.2%, C – 10.5% and the plasma pools at 3.7%.

The overall mean potency estimates for the functional activity, free antigen and total antigen of sample B, the proposed replacement standard are: 0.71, 0.83 and 0.88 IU/ampoule respectively (Table 8). The limited number of laboratories and range of different methods used for measuring each of the protein S analytes prevented any form of method comparison to be performed. However, within the data there were no outliers for any method/laboratory indicating that all assays were performed with good precision and accuracy.

The potency estimates of the locally sourced pools relative to the 2nd IS was generally more variable than for the supplied samples. Despite a diverse range of frozen plasma pools being used by participants, the geomean values were all close to 1 IU/ml – functional 1.00 IU/ml, free antigen 1.03 and total antigen 1.02 IU/ml. This confirmed the original definition of the International Unit and highlighted the stability of the current standard. The higher GCVs associated with the plasma pools (both intra and inter) relative to sample B (the proposed replacement) supports the continued use of an International Standard to ensure harmonisation of protein S measurement.

Proposal

Based on the results of this study, it is recommended that sample B, 22/202, should be the 3rd International Standard for Protein S Plasma with the following assigned values:

Function: 0.71 IU/ampoule,
Free antigen: 0.83 IU/ampoule
Total antigen: 0.88 IU/ampoule.

The instructions for use for the proposed International Standard, 22/202 is illustrated in appendix 6.

Participant Responses

All submitted responses from participants agreed with the proposals to establish 22/202 as the 3rd International Standard for Protein S, Plasma with the values as indicated above.

One comment was received:
Our laboratory had several unexpected analytical issues with the two commercial Protein S activity assays, the kind of which we have never experienced with similar studies on activities of coagulation factors II, X, VIII, IX, IXa, XI, and XII before. Specifically, we noted that plasma samples and international standards lacked parallelism and linearity even in the narrow range of sample dilutions, under all tested conditions and coagulation instruments available to us. Because of that, we think that some of the best practices or details about the Protein S activity assay analysis (which were used by the successful study participants) deserve to be explained in the study report, e.g., the use of slope ratio model.

It was commented to this lab that the models are described in the 1st reference, but it will be considered whether providing additional information beyond that which is described in the statistical analysis section will be useful.

ISTH SSC Expert Response

All experts (10 in total responded) from the International Society of Thrombosis and Haemostasis (ISTH) Scientific and Standardisation Committee (SSC) agreed with the proposal to establish 22/202 as the 3rd International Standard Protein S, Plasma. Several reviewers submitted comments which are indicated below with the response from MHRA.

Comment 1
According to ISO standard 15189 (2022) laboratories shall establish the measurement uncertainty of an examination procedure. This includes also the measurement uncertainty of the calibrator of reference plasma used. Commercial calibration plasmas are currently in most cases calibrated against the WHO standards. Manufacturers should take into account, for the calculation of the measurement uncertainty of their calibrator also the measurement uncertainty of the reference plasma of a higher order (e.g. WHO standard).
Unfortunately, SSC/WHO currently does not provide this information for their standards.
I therefore strongly recommend that SSC develops a policy to follow the intranational protocol for value assignment of reference plasmas (ISO 17511:2020).

Response
The International Standard, and associated International Unit, for this material (and others prepared for the WHO) are classified as the Highest Order material for the analyte described. As such, by definition WHO International Standards do not have an uncertainty applied to them in the manner as described in the indicated ISO guidelines above for the analytes that they represent. Any uncertainty associated with the material is limited to the coefficient of variation from the production process, which for this material was 0.13%.

Comment 2:
This is a well designed and stringently run study to replace the current protein S standard which is scheduled to run out in the near future. The proposed new standard (Sample B, NIBSC code 22/02) with assigned potencies for function, free antigen and total antigen, as outlined in the report is supported by the results and is endorsed as WHO reference material.

Response
We thank the reviewer for their comment.

Comment 3
The study is thorough and well designed, and it is valuable that there is a mix of manufacturer and diagnostic laboratory participants. With full recognition of the amount of work and costs involved to take part, and consequent difficulty to obtain participants, the number of participants is disappointingly but perhaps inevitably low. I also note there are no sites from Asia, Australasia, and South America. Having said that, they are similar in numbers to other reports I have seen, and multiple runs were performed, yet assigning a total protein S value from only 10 sources is a concern. Nonetheless, the results with pooled normal plasmas returning results close to 100%/1.0IU/mL gives confidence that the assays for each parameter were performing appropriately. Whilst high within-site CVs are unremarkable for manually performed ELISAs, occasional within-site CVs for automated functional assays were close to or above 10%, which is too high, but excluding them from the analysis would not have a significant impact on the final assigned values.

Response
We acknowledge the comment with regards to the spread and number of participants, which as the reviewer has pointed out will be challenging. This is something that we are keen to address and options for supporting laboratories to participate in future studies are being considered.

Comment 4:
Total PS antigen measurement was performed by ELISA by all labs. For free PS and functional testing methods were more diverse. Probably this clarifies the higher CV's for total PS antigen, along with the relative low number of participating labs.
Overall, the new standard with the assigned values can be accepted.

The analysis of data according to the commercial method for each test may be useful.
Appendix 3 appears in text before appendix 2

Response
We agree with the observation about the three different analyte measurements. The analysis of data was performed as denoted in the analysis section; however, it is likely that this comment relates to the fact that each commercial method will have a specific analysis procedure.

References

1) Campbell PJ. International biological standards and reference preparations. 1. Preparation and presentation of materials to serve as standards and reference preparations. J Biol Standardisation 1974; 2: 249-267
2) Recommendations for the preparation, characterization and establishment of international and other biological reference standards (revised 2004). In: WHO TRS, No. 932, 2006, Annex 2. pp.114-119 (section A.7)
3) Finney DJ. Statistical Method in Biological Assay. 3rd Edition. London: Charles Griffin 1978.
4) Grubbs F. Procedures for Detecting Outlying Observations in Samples. Technometrics, 1969; 11: 1-21.
5) Kirkwood T.B.L. (1977). Predicting the stability of biological standards and products. *Biometrics*, 33: 736-742.

Table 1a: Functional Activity: Potency estimates (IU/ampoule) from individual assays for sample B relative to the 2nd I.S. for Protein S, Plasma (sample A).

Lab	Assay				GM	GCV
	1	2	3	4		
3a	0.718	0.672	0.767	0.728	0.720	5.57%
3b	0.716	0.690	0.690	No assay	0.698	2.11%
3c	0.748	0.789	0.759	No assay	0.765	2.82%
4	0.679	0.647	0.663	0.668	0.664	2.03%
8	0.690	Invalid	0.657	0.797	0.712	10.57%
10	0.682	0.708	0.702	0.710	0.700	1.82%
12	0.738	0.699	0.731	0.772	0.735	4.19%
13a	0.675	0.666	0.725	0.689	0.688	3.81%
13b	0.675	0.663	0.645	0.661	0.661	1.90%
14a	0.725	0.739	0.738	0.708	0.728	2.03%
14b	0.825	0.739	0.679	0.787	0.755	8.78%
14c	0.720	0.779	0.738	0.790	0.756	4.49%
14d	0.727	0.712	0.772	0.679	0.722	5.46%
18	0.711	0.772	0.694	0.753	0.732	5.03%
19	0.678	0.677	0.705	0.692	0.688	1.96%
20	0.672	0.703	0.697	0.707	0.695	2.30%
Overall Geometric Mean (n=16)					**0.713**	
95% Confidence Limits					**0.697 - 0.730**	
Between-lab GCV					**4.48%**	

Table 1b: Functional Activity: Potency estimates (IU/ampoule) from individual assays for sample C relative to the 2nd I.S. for Protein S, Plasma (sample A).

Lab	Assay				GM	GCV
	1	2	3	4		
3a	0.630	0.614	0.645	0.623	0.628	2.12%
3b	0.624	0.617	0.618	No assay	0.620	0.57%
3c	0.667	0.714	0.698	No assay	0.693	3.49%
4	0.609	0.598	0.595	0.578	0.595	2.17%
8	0.589	Invalid	0.586	0.653	0.608	6.26%
10	0.597	0.609	0.613	0.621	0.610	1.63%
12	0.571	0.626	0.574	0.599	0.592	4.43%
13a	0.636	0.618	0.656	0.638	0.637	2.43%
13b	0.587	0.588	0.590	0.591	0.589	0.30%
14a	0.636	0.611	0.652	0.646	0.636	2.88%
14b	0.571	0.655	0.650	0.568	0.609	8.19%
14c	0.599	0.678	0.648	0.693	0.654	6.68%
14d	0.533	0.647	0.724	0.618	0.627	13.47%
18	0.626	0.682	0.636	0.663	0.651	3.95%
19	0.607	0.614	0.608	0.589	0.605	1.78%
20	0.611	0.632	0.630	0.618	0.623	1.57%
Overall Geometric Mean (n=16)					**0.623**	
95% Confidence Limits					**0.609 - 0.637**	
Between-lab GCV					**4.33%**	

Table 1c: Functional Activity: Potency estimates (IU/ampoule) from individual assays for locally included pools – P – relative to the 2nd I.S. for Protein S, Plasma (sample A).

Lab	Assay				GM	GCV
	1	2	3	4		
3a P1	0.941	0.923	0.961	0.965	0.947	2.11%
3a P2	0.958	0.860	0.949	0.917	0.920	5.01%
3b P1	1.076	1.028	1.017	No assay	1.040	3.05%
3b P2	1.063	0.959	0.995	No assay	1.005	5.35%
3c P1	1.026	0.983	0.977	No assay	0.995	2.66%
3c P2	0.864	0.996	0.923	No assay	0.926	7.35%
4	1.027	1.077	0.997	0.940	1.009	5.87%
8	0.963	Invalid	0.867	1.097	0.971	12.53%
10	0.995	1.050	1.036	1.044	1.031	2.47%
12	0.866	Invalid	0.937	1.093	0.961	12.59%
13a	0.838	0.979	0.888	0.951	0.912	7.25%
13b	0.937	1.043	0.897	1.054	0.980	8.32%
14a	1.081*	1.115	1.081*	1.037	1.078	3.03%
14b	1.111*	1.075	1.098*	1.156	1.109	3.10%
14c	0.926*	1.096	0.983*	1.064	1.015	7.95%
14d	0.960*	1.095	1.055*	1.025	1.032	5.72%
18						
19	0.803	1.121	1.023	1.110	1.005	16.85%
20						
Overall Geometric Mean (n=17)					**0.995**	
95% Confidence Limits					**0.968 – 1.02**	
Between-lab GCV					**5.56%**	

All assays included frozen pools except for lab 14 who used fresh and frozen pools – fresh indicated by *. Lab 3 included pools from two different sources (P1 and P2).

Table 2a: Free Antigen Activity: Potency estimates (IU/ampoule) from individual assays for sample B relative to the 2nd I.S. for Protein S, Plasma (sample A).

Lab	Assay				GM	GCV
	1	2	3	4		
1	0.844	0.890	0.866	0.772	0.842	6.39%
2	0.812	0.857	0.880	0.851	0.850	3.39%
3	0.763	0.802	0.785	0.847	0.799	4.47%
4	0.837	0.832	0.839	0.843	0.838	0.55%
6a	0.821	0.887	0.894	0.842	0.860	4.17%
6b	0.855	0.880	0.870	0.869	0.868	1.22%
7	0.852	0.772	0.813	0.829	0.816	4.22%
9	0.846	0.706	0.678	0.866	0.770	13.19%
10	0.861	0.866	0.850	0.848	0.856	1.05%
11	0.764	0.790	0.766	0.767	0.772	1.60%
12	0.744	0.743	0.788	0.771	0.761	2.90%
13a	0.835	0.867	0.830	0.834	0.841	2.06%
13b	0.880	0.846	0.853	0.839	0.854	2.12%
14a	0.777	0.805	0.773	0.799	0.788	2.03%
14b	0.822	0.848	0.789	0.841	0.825	3.31%
14c	0.807	0.914	0.830	0.959	0.875	8.38%
14d	0.899	0.816	0.830	0.885	0.857	4.88%
14e	0.860	0.847	0.921	0.926	0.888	4.71%
14f	0.880	0.899	0.774	0.817	0.841	7.12%
17	0.829	0.829	0.847	0.804	0.827	2.14%
18a	0.864	1.002	0.854	0.916	0.907	7.59%
18b	0.855	0.936	0.874	0.925	0.897	4.46%
19	0.758	0.788	0.796	0.809	0.788	2.81%
20	0.764	0.793	0.768	0.800	0.781	2.33%
Overall Geometric Mean (n=24)					**0.832**	
95% Confidence Limits					**0.815 - 0.850**	
Between-lab GCV					**5.15%**	

Table 2b: Free Antigen Activity: Potency estimates (IU/ampoule) from individual assays for sample C relative to the 2nd I.S. for Protein S, Plasma (sample A).

Lab	Assay				GM	GCV
	1	2	3	4		
1	0.745	0.813	0.738	0.719	0.753	5.52%
2	0.725	0.709	0.790	0.733	0.738	4.80%
3	0.658	0.719	0.630	0.699	0.676	6.09%
4	0.755	0.740	0.741	0.750	0.747	0.97%
6a	0.725	0.798	0.776	0.767	0.766	4.10%
6b	0.716	0.738	0.746	0.736	0.734	1.76%
7	Invalid	0.694	0.745	0.764	0.734	5.10%
9	0.757	0.666	0.550	0.770	0.680	16.84%
10	0.764	0.749	0.763	0.765	0.760	1.00%
11	0.671	0.671	0.656	0.670	0.667	1.12%
12	0.657	0.673	0.676	0.689	0.673	1.97%
13a	0.729	0.757	0.772	0.769	0.757	2.63%
13b	0.754	0.756	0.757	0.755	0.756	0.16%
14a	0.683	0.712	0.697	0.696	0.697	1.73%
14b	0.750	0.787	0.703	0.780	0.754	5.30%
14c	0.676	0.714	0.785	0.742	0.728	6.53%
14d	0.720	0.745	0.680	0.670	0.703	5.06%
14e	0.831	0.750	0.757	0.744	0.770	5.28%
14f	0.812	0.776	0.679	0.840	0.774	9.84%
17	0.709	0.737	0.739	0.740	0.731	2.05%
18a	0.792	0.874	0.815	0.791	0.817	4.81%
18b	0.730	0.778	0.773	0.768	0.762	2.93%
19	0.681	0.688	0.688	0.651	0.677	2.69%
20	0.689	0.713	0.676	0.694	0.693	2.25%
Overall Geometric Mean (n=24)					**0.730**	
95% Confidence Limits					**0.714 - 0.747**	
Between-lab GCV					**5.58%**	

Table 2c: Free Antigen Activity: Potency estimates (IU/ampoule) from individual assays for sample P relative to the 2nd I.S. for Protein S, Plasma (sample A).

Lab	Assay				GM	GCV
	1	2	3	4		
1						
2	1.214	1.191	1.063	1.053	1.128	7.71%
3 P1	0.925	0.985	0.995	0.929	0.958	3.88%
3 P2	0.903	0.985	1.047	0.967	0.974	6.30%
4	1.088	1.143	1.062	1.062	1.088	3.53%
6a	0.923	0.992	1.022	0.962	0.974	4.44%
6b	0.899	0.953	0.988	0.934	0.943	4.01%
7						
9	1.031	1.042	1.072	1.053	1.049	1.65%
10	1.122	1.130	1.118	1.135	1.126	0.70%
11	0.965	1.047	0.951	0.999	0.990	4.35%
12	1.199	1.217	1.145	1.231	1.198	3.24%
13a	0.903	1.074	0.932	1.067	0.991	9.46%
13b	0.997	1.084	0.927	1.099	1.024	8.24%
14a	1.066*	1.055	1.059*	1.009	1.047	2.53%
14b	0.950*	1.032	0.956*	1.056	0.997	5.51%
14c	1.100*	0.966	1.092*	1.092	1.061	6.46%
14d	1.093*	1.012	1.010*	0.939	1.012	6.39%
14e	0.924*	1.028	1.117*	1.008	1.017	8.08%
14f	0.904*	1.086	0.905*	1.095	0.993	11.41%
17						
18a						
18b						
19	0.965	1.064	1.057	1.101	1.045	5.81%
20						
Overall Geometric Mean (n=19)					**1.031**	
95% Confidence Limits					**1.002 – 1.066**	
Between-lab GCV					**6.39%**	

All assays included frozen pools except for lab 14 who used fresh and frozen pools – fresh indicated by *. Lab 3 included pools from two different sources (P1 and P2).

Table 3a: Total Antigen Activity: Potency estimates (IU/ampoule) from individual assays for sample B relative to the 2nd I.S. for Protein S, Plasma (sample A).

Lab	Assay				GM	GCV
	1	2	3	4		
3	0.87	0.86	0.87	0.89	0.87	1.25%
4	0.83	0.88	0.97	0.87	0.88	6.63%
11	0.78	Invalid	0.99	0.85	0.87	12.85%
14a	0.88	0.83	0.87	0.80	0.84	4.53%
14b	0.97	0.91	0.89	0.94	0.93	3.91%
15a	0.85	0.90	0.90	0.89	0.89	2.34%
15b	0.85	0.80	0.84	0.94	0.86	6.73%
16						
17	0.87	0.99	0.85	0.88	0.90	7.13%
18	0.91	1.01	0.86	0.90	0.92	7.26%
Overall Geometric Mean (n=9)					**0.884**	
95% Confidence Limits					**0.862 - 0.906**	
Between-lab GCV					**3.22%**	

Table 3b: Total Antigen Activity: Potency estimates (IU/ampoule) from individual assays for sample C relative to the 2nd I.S. for Protein S, Plasma (sample A).

Lab	Assay				GM	GCV
	1	2	3	4		
3	0.772	0.817	0.744	0.717	0.762	5.73%
4	0.843	0.758	0.790	0.790	0.795	4.49%
11	0.708	Invalid	0.725	0.899	0.773	14.09%
14a	0.793	0.719	0.813	0.788	0.777	5.55%
14b	0.815	0.812	0.909	0.799	0.833	6.08%
15a	0.905	0.930	0.959	0.914	0.927	2.58%
15b	1.074	1.042	0.946	1.073	1.032	6.21%
16						
17	0.765	0.876	0.735	0.844	0.803	8.58%
18	0.858	0.882	0.819	0.875	0.858	3.38%
Overall Geometric Mean (n=9)					**0.836**	
95% Confidence Limits					**0.774 - 0.903**	
Between-lab GCV					**10.52%**	

Table 3c: Total Antigen Activity: Potency estimates (IU/ampoule) from individual assays for sample P relative to the 2nd I.S. for Protein S, Plasma (sample A).

Lab	Assay				GM	GCV
	1	2	3	4		
3 P1	0.999	1.162	1.002	0.883	1.007	11.87%
3 P2	0.961	1.07	1.081	0.900	1.000	9.26%
4	0.976	1.054	1.135	1.102	1.065	6.76%
11	0.873	Invalid	0.973	1.058	0.965	10.14%
14a	1.038*	0.990	1.071*	1.128	1.056	5.61%
14b	1.051*	1.014	1.044*	0.949	1.014	4.77%
15a						
15b						
16						
17						
18						
Overall Geometric Mean (n=6)					**1.017**	
95% Confidence Limits					**0.979 – 1.037**	
Between-lab GCV					**3.71%**	

All assays included frozen pools except for lab 14 who used fresh and frozen pools – fresh indicated by *. Lab 3 included pools from two different sources (P1 and P2).

Table 4. Functional Activity of sample C relative to sample A, 2nd IS and relative to sample B with assigned value of 0.71 IU/ampoule.

Lab	Sample C			Pooled Plasma	
	Relative to A	Relative to B		Relative to A	Relative to B
3a	0.628	0.619		0.947/0.920*	0.974/0.959*
3b	0.620	0.630		1.040/1.005*	0.981/0.962*
3c	0.693	0.643		0.995/0.926*	1.023/0.927*
4	0.595	0.634		1.009	1.081
8	0.608	0.607		0.971	0.970
10	0.610	0.620		1.031	1.042
12	0.592	0.575		0.961	0.911
13a	0.637	0.656		0.912	0.945
13b	0.589	0.627		0.980	1.068
14a	0.636	0.621		1.078	1.051
14b	0.609	0.573		1.109	1.041
14c	0.654	0.613		1.015	0.957
14d	0.627	0.611		1.032	0.990
18	0.651	0.632			
19	0.605	0.624		1.005	1.038
20	0.623	0.637			
GM	**0.623**	**0.620**		**0.995**	**0.994**
GCV	**4.33**	**3.67**		**5.98**	**5.28**
P value	**0.624**			**0.923**	

*two different pools

Table 5. Free Antigen Activity of sample C relative to sample A, 2nd IS and relative to sample B with assigned value of 0.83 IU/ampoule.

Lab	Sample C			Pooled Plasma	
	Relative to A	Relative to B		Relative to A	Relative to B
1	0.753	0.743			
2	0.738	0.720		1.128	1.109
3	0.676	0.701		0.958/0.974*	1.006/1.109*
4	0.747	0.739		1.088	1.080
6a	0.766	0.737		0.974	0.941
6b	0.734	0.703		0.943	0.899
7	0.734	0.757			
9	0.680	0.733		1.049	1.131
10	0.760	0.737		1.126	1.092
11	0.667	0.717		0.990	1.067
12	0.673	0.731		1.198	1.320
13a	0.757	0.746		0.991	0.975
13b	0.756	0.734		1.024	0.997
14a	0.697	0.732		1.047	1.108
14b	0.754	0.759		0.997	1.008
14c	0.728	0.694		1.061	1.008
14d	0.703	0.680		1.012	0.995
14e	0.770	0.708		1.017	0.933
14f	0.774	0.763		0.993	0.978
17	0.731	0.732			
18a	0.817	0.749			
18b	0.762	0.706			
19	0.677	0.711		1.045	1.109
20	0.693	0.735			
GM	**0.730**	**0.727**		**1.031**	**1.037**
GCV	**5.58%**	**3.03%**		**6.39%**	**9.17%**
P value	**0.663**			**0.515**	

*two different pools

Table 6. Total Antigen Activity of sample C relative to sample A, 2nd IS and relative to sample B with assigned value of 0.88 IU/ampoule.

Lab	Sample C			Plasma Pools	
	Relative to A	Relative to B		Relative to A	Relative to B
3	0.762	0.767		1.007/1.000*	1.011/1016*
4	0.795	0.793		1.065	1.053
11	0.773	0.785		0.965	0.983
14a	0.777	0.809		1.056	1.121
14b	0.833	0.786		1.014	0.960
15a	0.927	0.918			
15b	1.032	1.058			
16					
17	0.803	0.785			
18	0.858	0.823			
GM	**0.836**	**0.832**		**1.017**	**1.023**
GCV	**10.52**	**11.01**		**3.71%**	**5.63%**
P value	**0.658**			**0.710**	

*two different pools

Table 7. Summary of intra-laboratory variability: number of mean estimates with GCVs less than 5%

Samples	Method Type		
	Functional	**Free Antigen**	**Total Antigen**
B	11/16 – 69%	19/24 79%	4/9 44%
C	12/16 – 75%	15/24 63%	3/9 33%
P	6/17 – 35%	9/19 50%	1/6 17%
Total	29/49 – 59%	43/65 66%	8/24 33%

Table 8. Summary of potency estimates for samples relative to A, 2nd I.S. for Protein S Plasma.

		Function	**Free Antigen**	**Total Antigen**
B (22/202)	Potency IU/amp GCV	0.71 4.5%	0.83 5.2%	0.88 3.2%
C	Potency IU/vial GCV	0.62 4.3%	0.73 5.6%	0.84 10.5%
P Normal Pools	Potency IU/ml GCV	1.00 5.6%	1.03 6.4%	1.02 3.7%

Figure 1 – Functional Potency Values for Sample B, C and pools relative to the 2nd I.S. for Protein S Plasma (Sample A) for each laboratory.

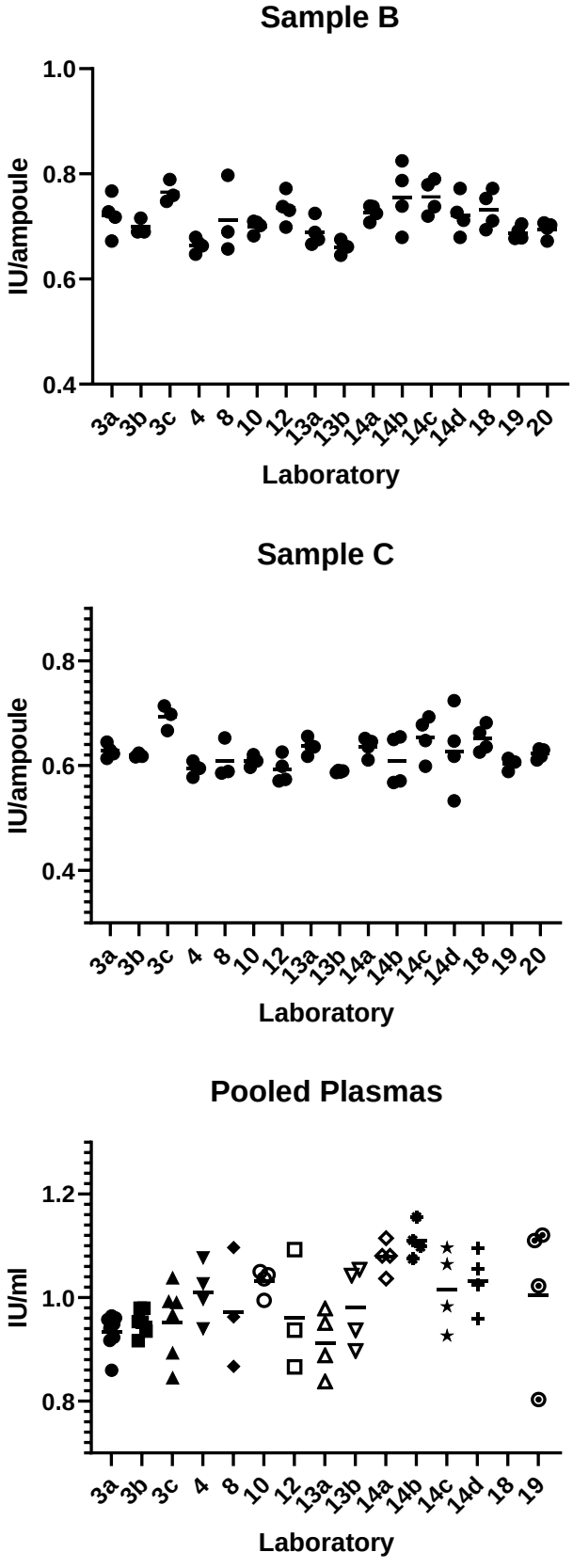


Figure 2 – Free Antigen Potency Values for Samples B, C and pools relative to the 2nd I.S. for Protein S, Plasma (Sample A) for each laboratory.

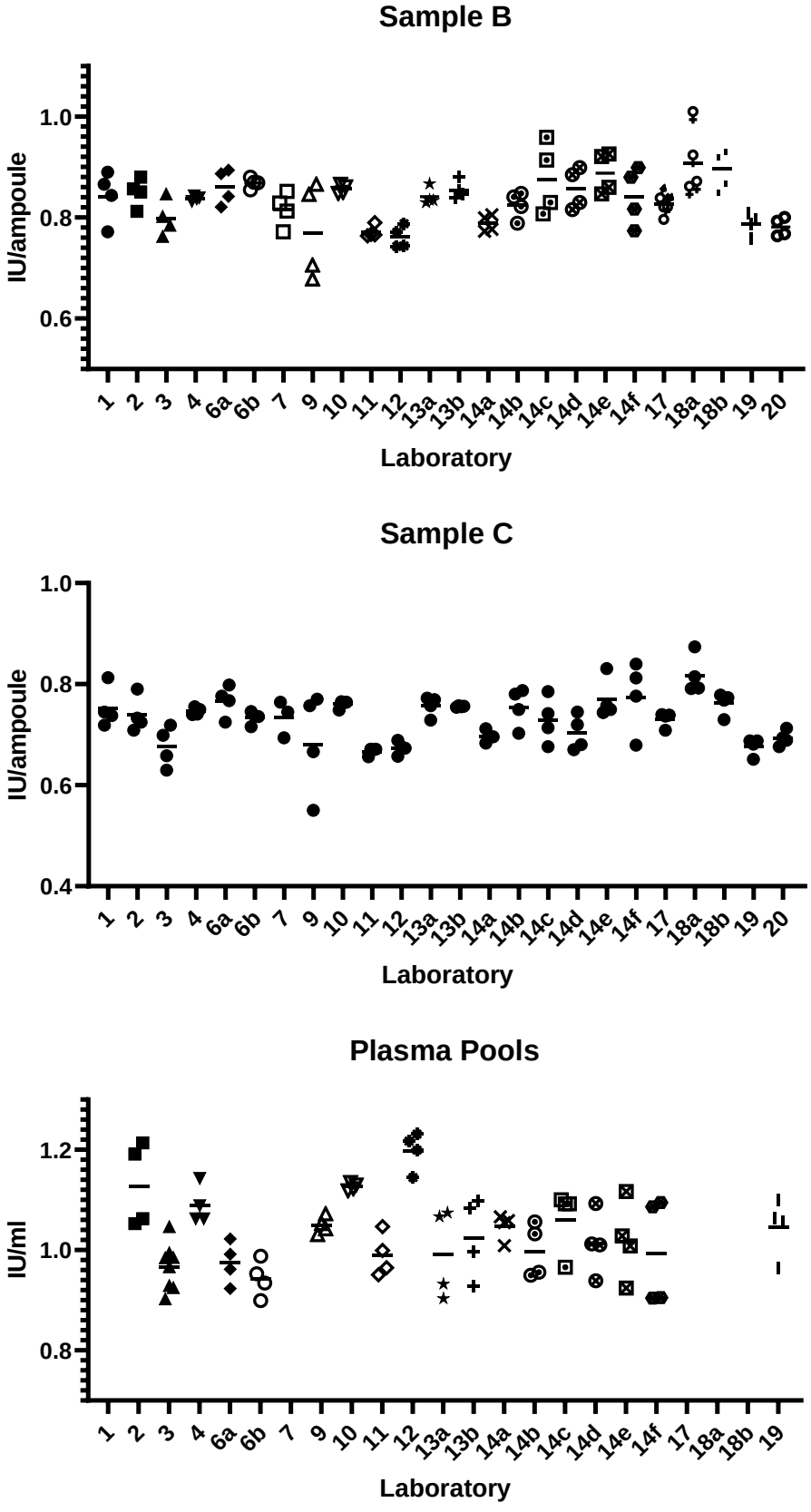
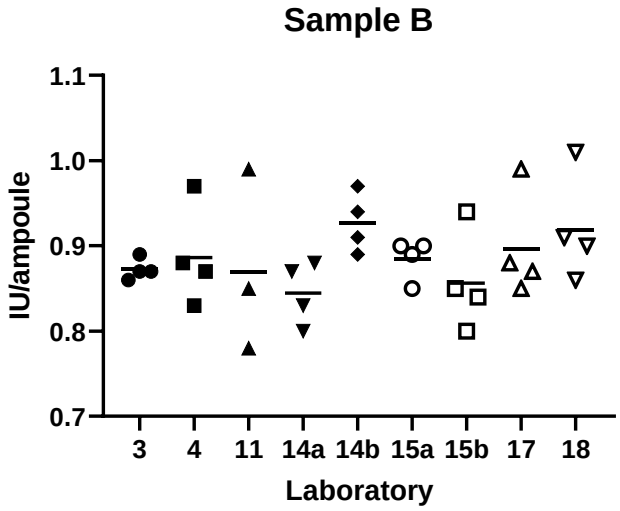
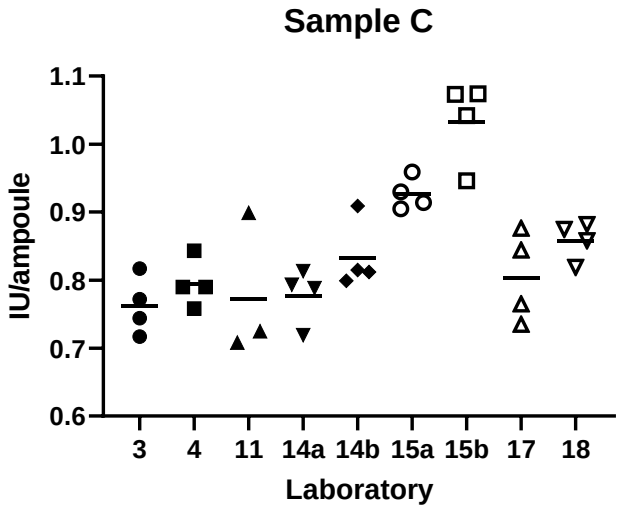
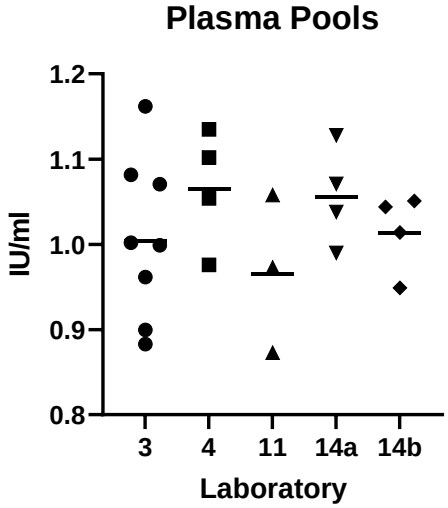


Figure 3 – Total Antigen Potency Values for Samples B, C and pools relative to the 2nd I.S. for Protein S, Plasma (Sample A). for each laboratory.







Appendix 1
List of participants

Kirsten Villadsen / Anders Abildgaard, Aarhus University Hospital, Aarhus N, DENMARK

Tammy Buckner, Corgenix, Inc., Broomfield, USA

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Peter Baker / Sarah Harper, Oxford Haemophilia and Thrombosis Centre, Oxford, GREAT BRITAIN

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Regina Gebauer / Dennis Gerlach / Michael Timme, Siemens Healthcare Diagnostics Products GmbH, Marburg, GERMANY

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Mandy Reinhardt / Martin Blum, Takeda Manufacturing Austria AG, Orth an der Donau, AUSTRIA

Mikhail Ovanesov / Stepan Surov / Ivan Tarandovskiy, CBER, U.S. Food and Drug Administration, Silver Spring, USA

Appendix 2

Product characteristics for the proposed 3rd IS for Protein S, Plasma, 22/202

	22/202
Presentation	Sealed, siliconized glass 2.5 ml DIN ampoules
Number of Ampoules available	8320
Liquid filling weight (g)	1.1078
CV of fill mass (%)	0.1275
Homogeneity by activity and free antigen (9 ampoules)	GCV = 1.87%, 1.67% (ANOVA = 0.433, 0.669)
Mean dry weight (g, n = 6)	0.0994 (CV = 0.74%)
Mean head space oxygen (%, n = 12)	0.51 (CV = 26.36%)
Residual moisture (%, n = 12)	0.416 (CV = 22.25%)
Manufacturing site	MHRA, South Mimms
Custodian	MHRA, South Mimms
Storage temperature	-20 °C

CV = coefficient of variation; GCV = geometric coefficient of variation

Appendix 3
Study Protocol

Protocol for the Collaborative Study to establish the WHO 3rd International Standard for Protein S, Plasma CS720

Aim of Study

The aim of the study is to assay the protein S, plasma candidate preparation against the 2nd International Standard, 03/228, with a view to establish the new material as the 3rd International Standard for protein S, plasma. Three analytes are associated with this standard.

Where feasible, participants are requested to include locally collected normal pooled plasma.

Samples Included

A – the 2nd International Standard (03/228) for protein S, plasma.
Functional – 0.77 IU/ampoule
Free antigen – 0.81 IU/ampoule
Total antigen – 0.83 IU/ampoule

B – proposed 3rd International Standard for protein S, plasma.
Functional – approximately 0.7 IU/ampoule
Free antigen – approximately 0.8 IU/ampoule
Total antigen – approximately 0.8 IU/ampoule

C – plasma sample
All activities approximately 0.6 IU/ampoule

The samples should be handled as follows:

1. Store all unopened ampoules at or below -20°C.

2. On day of testing, allow the ampoules to warm to room temperature (about 10 min). Prior to opening ensured all the contents are in the lower half by gentle tapping, then open and reconstitute each with 1.0 ml distilled water. Allow the ampoules to stand for 10 minutes at room temperature and aid reconstitution by regular gentle swirling. Transfer the entire contents to stoppered plastic tubes and stored on melting ice.

Local Normal Pooled Plasma – if included

Collect fresh plasma on two separate days to prepare pools F_1 and F_2. The method of collections for the fresh normal plasma is a key part of the study and should be broadly standardised as far as possible, with a suggested method as below. If freshly prepared normal pooled plasma cannot be prepared on day of assay, please use different batches of frozen normal pools.
Donors – Normal healthy volunteers, excluding pregnant women and women taking oral contraceptives. Take blood from a minimum of 8 different donors for each pool on each day.
Anticoagulant – 0.109 mol/L tri-sodium citrate or a mixture of tri-sodium citrate and citric acid with a total concentration of 0.109 mol/L. Add 9 volumes of blood to 1 volume of anticoagulant.
Centrifugation – Blood should be centrifuged at 4ºC as soon as possible after collection either at 50,000g for 5 minutes or at 2,000 g for 20 minutes.
Storage – Keep the pooled plasma in a plastic stoppered tube at 4ºC during the assay period. Snap freeze aliquots for further testing. When required thaw at 37ºC and store on melting ice (thawed plasma indicated as FF_1 and FF_2).

Study Protocol

Each laboratory is requested to perform its in-house method(s) for protein S in the manner as described below. If you have any questions about the design of the study, please do contact prior to testing on the email below.

Design and Number of Assays

Samples: Samples A, B and C coded ampoules (dispatched from MHRA) and F_1/F_2 and FF_1/FF_2 are locally collected pools. A fresh ampoule should be used for each assay. Where possible, if you have indicated measurement of more than one analyte, the testing should be performed on the same set of samples where feasible. An additional two sets of samples have been included for laboratories who have indicated that they will perform functional and antigen-based determinations.
Please note due to limited stocks of the International Standard, further samples cannot be supplied.

Number of assays: All participants are requested to perform 4 independent assays, preferably on separate days, for each type of method – additional methods per analyte are most welcome.

Assay design: All samples should be included in each of the 4 assays. A minimum of three dilutions of each preparation should be tested, in replicate, within each assay. Please follow a balanced assay design such as the 8-place assay described below. In the following design each letter represents three or more dilutions of a sample; where A*, B* etc indicates a separately prepared set of dilutions (replicates). Duplicate measurements for each dilution can be included if so wished.

8-place assays:

Assay 1:	A	B	C	F_1	F_1*	C*	B*	A*
Assay 2:	B	F_2	A	C	C*	A*	F_2*	B*
Assay 3:	FF_1	C	B	A	A*	B*	C*	FF_1*
Assay 4:	C	FF_2	A	B	B*	A*	FF_2*	C*

If you require assistance with setting up a balanced assay design or are unsure of the assay design request as above, please send an email to the address below.

Report of data:

Raw data, calculated estimates and assay information should be recorded in the excel workbook and returned electronically to John Hogwood by email john.hogwood@nibsc.org. Data should be returned within two months (around 31st March) of sample receipt for inclusion in the analysis.

Appendix 4

Methods used by the participants.

Lab	Functional	Free Antigen	Total Antigen
1		Asserchrom ELISA	
2		Siemens Innovance	
3	Werfen HemosIL x 2 and Stago	Corgenix ELISA	Corgenix ELISA
4	Hyphen	Hyphen Latex	Hyphen ELISA
5	No data returned		
6		MRX Blue and MRX Red from Nordic Biomarker	
7		Stago Latex	
8	Werfen		
9		Siemens Innovance	
10	Werfen	Siemens Innovance	
11		Werfen Latex	Affinity Biologicals
12	Precision Biologicals	Werfen Latex	
13	Siemens Protein S Ac on different coagulometers	Siemens Innovance on different coagulometers	
14	Werfen, Hyphen, Stago & Siemens	Werfen, Hyphen, Stago & Siemens Latex with Hyphen and Asserchrom ELISAs	Hyphen and Asserchrom ELISAs
15			Inhouse and Asserchrom ELISAs
16			Asserchrom ELISA
17		Corgenix ELISA	Corgenix ELISA
18	Stago	Stago Latex and Asserchrom ELISA	Asserchrom ELISA
19	Werfen	Werfen Latex	
20	Werfen	Werfen Latex	
21	No data returned		
Total	**16**	**24**	**10**

Appendix 5

Predicted stability at elevated temperatures for the functional activity of protein S, plasma in 22/202

Temperature	% loss per year	95% UCL loss per year
-150ºC	0	0
-70ºC	0	0
-20ºC	0.013	0.133
+4ºC	0.549	3.349
+20ºC	4.711	17.272
+37ºC	31.541	50.395

Appendix 6 Draft IFU

Medicines & Healthcare products Regulatory Agency

NIBSC Confidence in Biological Medicines

WHO International Standard
3rd International Standard Protein S, Plasma
NIBSC code: 22/202
Instructions for use
(Version [Q-DOCS_Version], Dated [Q-DOCS_Date_Published])

9

1. INTENDED USE

The 3rd International Standard for Protein S, Plasma, Human consists of ampoules (code-labelled 22/202) containing 1 ml freeze-dried pooled fresh huan plasma. This standard has been assigned potencies for free and total Protein S antigen and for Protein S functions.

2. CAUTION

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. This preparation is not for administration to humans or animals

3. UNITAGE

The 3rd International Standard was calibrated in an international collaborative study involving 21 laboratories for Free Protein S antigen, Total Protein Antigen and Protein S function by assay against the 2nd International Standard (03/228). The standard was established by the WHO Expert Commeitte on Biological Standardisation in October 2023. Details of the collaborative study are avaliable in the WHO document WHO/BS/2023.XXX.
The following values have been assigned to the 3rd Internaional Standard:

Protein S function:	0.71 IU per ampoule
Free Protein S antigen:	0.83 IU per ampoule
Total Proteins S antigen:	0.88 IU per ampoule

Uncertainty: The International Unit of 22/202 is assgned without uncertainty. The uncertainty of the ampoule content of 22/202 may be considered to be the coefficient of variation of the ampolue filling, which was determined to be 0.13%

4. CONTENTS

Country of origin of biological material: United Kingdom.
The 3rd International Standard was prepared in September 2022 from a plasma pool collected from 30 noraml heathy donors. Blood was collected into CPD-adenine anticoagulant into plastic packs, at a ratio of 450 ml to 63 ml anticoagulant. Each donation underwent leuko-filtration and was double spun before storage at -70°C. The units were thawed in a 37°C water bath and pooled on the day of filling. THe final pool was buffered by the addition of HEPES (N-[2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid]) to a final concentration of 40 mmol/L and Glycine to a final concentration of 1% w/v with the plasma diluted by 10%.

Distribution into Ampoules

The pooled plasma was kept at 4°C throughout distribution into approximately 8,000 ampoules, then freeze-dried acording to the requirements for International Biological Standards (1). The cofficient of variation for the liquid fill was 0.13% and the fill weight was 1.1078 (range 1.1000 to 1.1150 g) to correct for the dilution by HEPES/Glycine buffer. The final content of the freeze-dried material has a mean dry weight of 0.099g and mean residual moisture of 0.42%

5. STORAGE

Unopend ampoules should be stored at -20°C or below.
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. Various types of ampoule breaker are available commercially. To open the ampoule, tap the ampoule gently to collect material at the bottom (labelled) end and follow manufactures instructions provided with the ampoule breaker.

7. USE OF MATERIAL

No attempt should be made to weigh out any portion of the freeze-dried material prior to reconstitution
Allow the ampoules to warm to room temperature. Reconstitute the total contents with 1.0 ml of distilled water and allow to standr for 10 minutes at room temperture and aid reconstitution by regular gentle shaking. Transfer the reconstitted contents to a plastic tube and keep on melting ice. Under these conditions the stanadrd has been found to be sufficient stable for use over a 4 hour period, however it is recommended to use the marterial once reconstituted.
Storage of the reconstituted standard under different conditions must be validated locally by users.

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 the WHO not to assign expiry dates to International Reference materials. They remain valid with the assigned potency and status until wthdrawn or amended.

Accelerated degradation studies, which involve potency estimation of ampoules stored at elevated temperatures relative to ampoules stored at below -70°C, and have shown that the material is very stable in unopened ampoules stored at -20°C. Predicted loss over one year whlst stored at -20°C is less than 0.1%.

9. REFERENCES

1. Campbell PJ (1974) J Biol Standardisation, 2 249-267

10. ACKNOWLEDGEMENTS

The efforts of the participants in the collaborative study, the members of the Plasma Coagulation Inhibitors sub-committee and the staff of the Centre for Biological Reference Materials (NIBSC) are gratefully acknowledged

11. FURTHER INFORMATION

Further information can be obtained as follows;
This material: enquiries@nibsc.org
WHO Biological Standards:
http://www.who.int/biologicals/en/
JCTLM Higher order reference materials:

National Institute for Biological Standards and Control,
Potters Bar, Hertfordshire, EN6 3QG. T +44 (0)1707 641000, nibsc.org
WHO International Laboratory for Biological Standards,
UK Official Medicines Control Laboratory

World Health Organization

Medicines & Healthcare products Regulatory Agency

NIBSC Confidence in Biological Medicines

http://www.bipm.org/en/committees/jc/jctlm/
Derivation of International Units:
http://www.nibsc.org/standardisation/international_standards.aspx
Ordering standards from NIBSC:
http://www.nibsc.org/products/ordering.aspx
NIBSC Terms & Conditions:
http://www.nibsc.org/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.org

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

Classification in accordance with Directive 2000/54/EC, Regulation (EC) No 1272/2008: Not applicable or not classified

Physical and Chemical properties			
Physical appearance: Solid		Corrosive:	No
Stable:	Yes	Oxidising:	No
Hygroscopic:	Yes	Irritant:	No
Flammable:	No	Handling: See caution, Section 2	
Other (specify):	PContains material of human origine		
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

In the event that this document is translated into another language, the English language version shall prevail in the event of any inconsistencies between the documents.
Unless expressly stated otherwise by NIBSC, NIBSC's Standard Terms and Conditions for the Supply of Materials (available at http://www.nibsc.org/About_Us/Terms_and_Conditions.aspx or upon request by the Recipient) ("Conditions") apply to the exclusion of all other terms and are hereby incorporated into this document by reference. The Recipient's attention is drawn in particular to the provisions of clause 11 of the Conditions.

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: 99 mg
Toxicity Statement: Non-toxic
Veterinary certificate or other statement if applicable. **Attached:** No

17. CERTIFICATE OF ANALYSIS

NIBSC does not provide a Certificate of Analysis for WHO Biological Reference Materials because they are internationally recognised primary reference materials fully described in the instructions for use. The reference materials are established according to the WHO Recommendations for the preparation, characterization and establishment of international and other biological reference standards http://www.who.int/bloodproducts/publications/TRS932Annex2_Inter_biolefstandardsrev2004.pdf (revised 2004). They are officially endorsed by the WHO Expert Committee on Biological Standardization (ECBS) based on the report of the international collaborative study which established their suitability for the intended use.

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Potters Bar, Hertfordshire, EN6 3QG. T +44 (0)1707 641000, nibsc.org
WHO International Laboratory for Biological Standards,
UK Official Medicines Control Laboratory

World Health Organization