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CALIBRATION OF THE PROPOSED WHO 1st INTERNATIONAL STANDARD FOR BLOOD COAGULATION FACTOR V IN PLASMA, HUMAN (03/116)

Anthony R Hubbard and *Suzanne Johnes

Division of Haematology and *Biostatistics Section, National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Potters Bar, Herts. EN6 3QG United Kingdom

SUMMARY

An international collaborative study involving 22 laboratories in 11 different countries has been undertaken to calibrate the Proposed WHO 1st International Standard Factor V Plasma (coded 03/116) for Factor V clotting activity (FV:C). Calibration of the Proposed WHO 1st IS was based on assays of FV:C relative to locally collected normal plasma pools. Most estimates (21/23) were obtained using thromboplastin-based methods rather than APTT-based methods (2/23). Thirteen estimates were obtained relative to fresh pools (range 0.62 – 0.81 unit/ampoule, mean 0.74 unit/ampoule) and 9 estimates relative to frozen pools (range 0.69 - 0.94 unit/ampoule, mean 0.80 unit/ampoule). Estimates calculated relative to the fresh pools were significantly lower than estimates calculated relative to the frozen pools (p=0.047) and this could indicate that some degradation of FV had occurred during freeze/thawing of the frozen pools. Following the precedent set for the calibration of Factor VIII clotting activity in plasma it is therefore proposed that the calibration should be based only on the estimates relative to the fresh local pools with an assigned mean value of 0.74 IU/ampoule from 13 estimates and inter-laboratory variability (geometric coefficient of variation, GCV) of 7.63 %.

The influence of the Proposed WHO 1st IS on inter-laboratory variability was evaluated through the estimation of FV:C in a common lyophilised plasma sample (SSC/ISTH Secondary Coagulation Standard Lot #3) relative to the Proposed WHO 1st IS. Twenty three estimates were obtained comprising 21 thromboplastin-based methods and 2 APTT-based methods. There was very good agreement between the 23 estimates with inter-laboratory variability (GCV) of 3.55% and a range of estimates from 110 - 124% of the proposed assigned value of the Proposed WHO 1st IS. There was also good agreement between results from APTT- and thromboplastin-based methods indicating that the Proposed WHO 1st IS will be suitable for use with both methodologies.

Predictions of the stability of FV:C in the Proposed WHO 1st IS, based on an accelerated degradation study in 3 laboratories, indicated a loss of less than 0.01 % per year at -20 ^{o}C. This indicates that the Proposed WHO 1st IS is extremely stable and suitable for prolonged use.

PROPOSAL

It is proposed that the WHO 1st IS Factor V Plasma (03/116) be assigned a value based on the estimates calculated relative to the fresh normal plasma pools of 0.74 IU Factor V:C per ampoule.

PRODUCT SUMMARY FOR THE PROPOSED WHO 1st IS Factor V Plasma (03/116)	
Presentation	**sealed glass DIN ampoules**
Excipients/additives	**1. Citrate-phosphate-dextrose-adenine anticoagulant (CPD-A) 2. HEPES buffer 40 mmol/L (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid)**
Coefficient of variation of the liquid fill (%)	**0.05 % based on 61 check-weight ampoules**
Liquid filling weight (g)	**mean 1.0052 g (range 1.0038 g - 1.0066 g)**
Residual moisture after lyophilisation and secondary desiccation (%)	**mean 0.152 % (n = 6)**
Dry weight (g)	**mean 84.0 mg (n = 6)**
Reconstitution volume and fluid	**1.0 ml of distilled water**
Number of ampoules in stock	**5,260**

Note on unitage: the term “unit” has been used where potencies are estimated relative to the local normal plasma pools which were arbitrarily assigned a value of 1.0 unit/ml. The term “International Unit” (IU) has been used for the proposed assigned potency of the Proposed WHO 1st IS and for potencies estimated relative to this proposed assigned potency.

1 INTRODUCTION AND OBJECTIVES OF THE STUDY

Estimation of Factor V clotting activity (FV:C) in human blood plasma is performed for several reasons. These include the diagnosis of rare congenital bleeding disorders such as FV deficiency ("*parahaemophilia*") and combined FV/FVIII deficiency, as well as investigations into coagulation disorders linked to liver disease. The need to review the management of rare bleeding disorders, including improved standardisation of diagnostic coagulation tests, has recently been highlighted (1). Factor V:C estimation is also a requirement for the quality control of a therapeutic product, virus-inactivated fresh frozen human plasma, as described in the European Pharmacopoeia (2). Despite the existence of several commercial reference plasmas with assigned values for FV:C there is currently no internationally accepted unit (IU). Consequently the commercial reference plasmas are calibrated independently of each other and there is evidence from external quality assurance surveys that this can lead to considerable differences between laboratories (3). The primary objective of the present exercise is to calibrate the Proposed WHO 1st International Standard for Factor V in Plasma with an internationally agreed value in International Units (IU). This standard will then be available for the calibration of commercial reference plasmas and other secondary standards with a view to improving harmonisation in FV estimation. This standard is intended to be used in the *in vitro* diagnostics field and relates to case 4 of the principles set out in ISO 17511.

In common with the principle adopted for most plasma coagulation factors (eg factors II, VII, VIII, IX, X, XIII) the calibration has been based on a relative comparison with locally collected normal plasma pools which were arbitrarily assigned a value of 1.0 unit per ml. This approach allows the International Unit (IU) to approximate to the population mean value for FV:C and so facilitates the definition and understanding of the normal and pathological ranges. The large majority of laboratories estimate FV:C using clotting methods involving activation of the extrinsic system of coagulation via the use of thromboplastin-reagents; a much smaller number of laboratories use methods involving activation of the intrinsic system of coagulation via the use of APTT reagents. Both methods have been included in this study and the relative numbers using each method reflect the use in the wider scientific community.

The work schedule was presented to, and approved by, the SSC/ISTH sub-committee on FVIII during the session on "Rare Bleeding disorders" in July 2004. Subsequently, the proposal was included on the WHO schedule of approved projects in November 2004.

A second freeze-dried plasma (SSC/ISTH Secondary Coagulation Standard Lot #3) was also included in this calibration exercise in addition to the candidate WHO material. This sample consists of a freeze-dried pooled normal plasma in rubber-sealed glass vials and is a secondary plasma standard prepared under the auspices of the SSC/ISTH Working Group on Coagulation Standards. Inclusion of this sample not only allowed for its calibration for FV:C but also provided an opportunity to evaluate the effect of the Proposed WHO 1st IS on the inter-laboratory variability of FV:C estimation by both types of clotting methodology, in a common test sample. This comparison also addresses the issue of commutability for the primary use of the Proposed WHO 1st IS in the calibration of secondary reference plasmas using many different reagent/instrument combinations.

Some laboratories also measured FV using other methods (chromogenic, antigen); however these are included for information only since there is insufficient data for formal calibration of these parameters.

2 MATERIALS

2.1 Candidate WHO 1st International Standard Factor V, Plasma (code number 03/116)

Bulk Material The Proposed WHO 1st IS was prepared from a plasma pool derived from 26 normal healthy donors (United Kingdom Blood Service, North London Blood Transfusion Centre). Blood was collected using conventional venepuncture into CPD-adenine anticoagulant at a nominal ratio of 63 ml anticoagulant to 450 ml whole blood. The donations underwent leuko-filtration followed by two centrifugation steps after which the plasma was stored at -70 ^{o}C until the day of ampoule filling. On the morning of the fill the plasma units were thawed in a waterbath at 37 ^{o}C and pooled. A buffering agent HEPES (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid) was added to the pooled plasma at a final concentration of 40 mmol/L.

Safety Testing Individual donations were tested and found negative for HBsAg, antibodies to HIV-1 and -2 and antibodies to HCV. The donations were also tested as mini-pools and found negative for the presence of HCV RNA using a PCR technique.

Ampoule filling Filling was carried out on 22 May 2003 by the Centre for Biological Reference Materials, NIBSC, Potters Bar, UK. Each glass ampoule received 1 ml of the pool, followed by freeze-drying and secondary desiccation according to the requirements for International Biological Standards (4). The ampoules were sealed in an atmosphere of dry nitrogen gas and placed into storage at -20 ^{o}C on 2 June 2003. The precision of the liquid fill was estimated using 61 check-weight ampoules spaced evenly throughout the fill. The mean fill weight was 1.0052 g and the range was 1.0038 - 1.0066 g with a coefficient of variation of 0.05 %.

Tests on the freeze-dried material Mean dry weight from 6 estimates was 0.0840 g. Residual moisture was estimated using Karl Fischer titration and a mean of 0.152 % was calculated from 6 estimates.

Number of ampoules and storage A total of 5,260 ampoules are stored at -20 ^{o}C at the National Institute for Biological Standards and Control, Potters Bar, UK.

2.2 SSC/ISTH Secondary Coagulation Standard Lot #3

The SSC/ISTH Secondary Coagulation Standard Lot #3 (SSC Lot #3) was prepared by a commercial manufacturer from a pool of 55 litres of normal plasma collected using apheresis. SSC Lot #3 consists of rubber-sealed, screw-capped vials each containing 1 ml of pooled normal plasma, freeze-dried.

2.3 Coding of the samples

The following coded samples were included in the study:

A Proposed WHO 1st IS Factor V Plasma (03/116)

B SSC/ISTH Secondary Coagulation Standard (Lot #3)

N_1, N_2 Normal plasma pools collected locally (see Appendix 1 for instructions on the method of preparation).

3 COLLABORATIVE STUDY

3.1 Participants

A total of 22 laboratories from 11 different countries participated in the study, each of which has been assigned a code number which does not reflect the order of listing in Appendix 2. The participants comprised 9 manufacturers of diagnostic calibrant plasmas or plasma-derived therapeutics, 10 clinical laboratories and 3 regulatory institutes.

3.2 Assay methods

Laboratories were requested to use their routine assay methodology as far as possible and to follow balanced assay designs as described in the study protocol (Appendix 1). Details of the reagents and instruments used by each individual laboratory are given in Table 1. In summary, Factor V clotting activity (FV:C) was estimated in 21 laboratories using thromboplastin-based methods employing 7 different thromboplastin reagents and 8 different instrument types. Two laboratories estimated FV:C using APTT-based methods. Factor V activity was also estimated using an "in house" chromogenic method in 1 laboratory and Factor V antigen was measured, using ELISA techniques, in 3 laboratories.

3.3 Study design

Participants were requested to carry out four independent assays for each method using fresh samples of A and B and locally collected normal plasma pools (N_1 and N_2) in each assay. It was requested that N_1 be included in assays 1 and 2 and N_2 be included in assays 3 and 4. Where a laboratory used more than one method it was necessary to use the same samples for both methods.

Estimates of Factor V clotting activity were performed on freshly reconstituted samples of A and B and, if possible, fresh normal plasma pools (N_1 and N_2). Thirteen out of the 21 laboratories performed FV clotting assays using fresh plasma pools prepared from a total of 512 donors; 12 of these laboratories used between 12 to 41 different donors to prepare N_1 and N_2 and one laboratory used 262 donors to prepare the pools. Nine laboratories performed FV clotting assays using frozen pools prepared from a total number of donors exceeding 1500.

Participants were allowed to use frozen aliquots of A, B and N for the estimation of FV antigen. All 3 laboratories estimating FV antigen used frozen plasma pools.

3.4 Statistical analysis

Assays were analysed as parallel line bioassays (5), relating assay response to log concentration. This analysis requires linear response (or transformed response) lines, when plotted against log concentration, which are parallel for all preparations included in the assay. All results were plotted and the validity of the assays assessed both visually and by analysis of variance. In most cases all of the returned data was included in the construction of the dose-response relationships and yielded satisfactory linearity and parallelism. In a small number of cases some data points at the extreme ends of the dose-response relationships were deleted to achieve linearity. Subject to this minor manipulation all results were considered valid and included in the study.

Where a laboratory performed more than one assay method, or an additional variation of the same assay method, each was treated as if it was performed by different laboratories.

Variability within laboratories (between assays) and between laboratories was measured by calculating geometric coefficients of variation (% GCV's) (6). Results of assays both within and between laboratories were combined to give the geometric mean. Detection of outlying results was performed using Duncan's test (7).

4 RESULTS

4.1 Number of assays returned

A total of 90 assays for FV:C in the Proposed WHO 1st IS were returned for analysis comprising 82 thromboplastin-based assays and 8 APTT-based assays; each individual laboratory returned 4 assays as requested except for Laboratory 10 which returned only 2 assays. In addition, a total of 9 assays of FV:antigen and 4 assays of FV chromogenic activity were returned.

4.2 Assay data and validity

Significant deviations from parallelism, at the 1% level, were found for 6 of the assays and significant deviations from linearity, at the 1% level, were found for 12 of the dose-response relationships. However, visual inspection of these assays and samples indicated that the apparent significant deviations were caused by very close agreement between replicate dilutions and hence these assays were not excluded.

Satisfactory parallelism of the dose-response relationships between the Proposed WHO 1st IS (A), the normal pools (N) and SSC Lot #3 (B) was confirmed by calculation of the slope ratios. A maximum ratio range of 0.91 – 1.10 for individual laboratories and an overall mean ratio of 1.00 supported the decision to include all assays in the analysis.

4.3 FV:C in the Proposed WHO 1st IS (A) vs Normal Pools (N_1, N_2)

Mean estimates from the individual laboratories ranged from 0.62 to 0.94 unit/ampoule and estimates of intra-laboratory (between assay) variability (GCV) ranged from 0.86% to 17.97% (Table 2a and Figure 1). There were no outlying results detected using Duncan's test. Estimates obtained relative to fresh normal pools (n=13) ranged from 0.62 to 0.81 unit/ampoule with an overall mean of 0.74 unit/ampoule and inter-laboratory variability (GCV) of 7.63 %. Estimates calculated relative to the frozen normal pools (n=9) ranged from 0.69 to 0.94 unit/ampoule with an overall mean of 0.80

unit/ampoule and inter-laboratory variability (GCV) of 10.14 %. Estimates relative to the fresh pools were significantly different to estimates relative to the frozen pools at the 5% level (t-test p=0.047). Combination of all 22 estimates gave a mean of 0.77 unit/ampoule and inter-laboratory variability (GCV) of 9.43 %.

4.4 Estimates of Factor V in the Proposed WHO 1st IS by other methods

One laboratory (number 20) used an "in house" chromogenic method to estimate FV activity. The mean estimate, relative to a frozen plasma pool, from 4 assays was 0.80 unit/ampoule with intra-laboratory variability (GCV) of 6.35%.

Three laboratories estimated FV antigen in a total of 9 assays and all estimates were made relative to frozen plasma pools. The overall mean estimate from the 3 laboratories was 0.84 unit/ampoule with inter-laboratory variability (GCV) of 7.08% (Table 2b).

4.5 Use of the Proposed WHO 1st IS to estimate FV:C in a common test sample (SSC/ISTH Secondary Coagulation Standard Lot #3) in multiple laboratories

Estimates of SSC Lot #3 were calculated relative to a nominal value of 100 % assigned to the Proposed WHO 1st IS. Twenty three estimates were obtained and these ranged from 110% to 124% of the Proposed WHO 1st IS (Table 3 and Figure 2). Intra-laboratory variability (GCV%) ranged from 1.04 to 16.14%. Combination of all estimates yielded an overall mean of 118% with inter-laboratory variability (GCV) of 3.55%. Estimates obtained by the two laboratories using the APTT-based methods agreed well with the estimates obtained using the thromboplastin-based methods.

5 VARIABILITY OF THE LOCAL NORMAL PLASMA POOLS

Estimates of the FV:C content in the different local normal pools were calculated relative to the Proposed WHO 1st IS using the proposed assigned value of 0.74 IU/ml (Table 4 and Figure 3). Estimates ranged from 0.91 - 1.19 IU/ml for the fresh pools (n=13) with a mean of 1.00 IU/ml and from 0.79 – 1.07 for the frozen pools (n=9) with a mean of 0.92 IU/ml. There was a significant difference in FV:C content between the fresh and frozen pools at the 5% level (t-test p=0.047).

6 STABILITY STUDIES

6.1 Accelerated degradation study

Stability of the Proposed WHO 1st IS has been assessed in an accelerated degradation study which involves the potency estimation of ampoules stored at elevated temperatures (4, 20, 37, 45 ^{o}C) relative to ampoules stored at the bulk storage temperature of -20 ^{o}C. The observed relative loss of potency is analysed using the Arrhenius equation in order to provide a prediction of loss per year for ampoules stored at various temperatures (8).

Three laboratories performed the testing on ampoules stored for either 4 months (37, 45 ^{o}C) or 22 months (4, 20 ^{o}C) and their results are presented in Table 5. Estimates of predicted loss per year for storage at -20, +4 and +20 ^{o}C are presented in Table 6.

Results from all three laboratories are consistent with a predicted loss of FV:C below 0.01 % per year for ampoules at the bulk storage temperature of -20 ^{o}C. This indicates that the preparation is exceedingly stable and suitable for long term use as an International Standard. The predicted loss for samples stored at +20 ^{o}C ranged from 6 to 14 % per year and this supports the shipment of ampoules at ambient temperature. The accelerated degradation study will continue for the lifetime of the standard.

6.2 Stability after reconstitution

Although the Instructions for Use will recommend that assays are performed as soon as possible after reconstitution it is useful to indicate a suitable period of use. The stability of coagulation factors in plasma standards, after reconstitution, is mainly affected by two components - the surface of the container and the storage temperature. In common with previous WHO Plasma Standards for blood coagulation factors it is recommended that the standard is transferred, after reconstitution, to a plastic tube in order to prevent activation by the glass surface of the ampoule. Recommendations for the storage after reconstitution have been limited to the period of storage on melting ice since local ambient temperature can vary considerably. Results from two separate tests, performed at NIBSC, indicated that 97% and 102% respectively of the starting concentration of FV:C was retained after 2 hours for the freshly reconstituted standard when stored in melting ice in plastic tubes. This period is sufficient for numerous assays of FV:C to be performed. Users will be advised that local validation will be necessary if the reconstituted standard is stored under different conditions.

The use of frozen aliquots of the Proposed WHO International Standard cannot be recommended since the effect of freezing and thawing, under local conditions, on the FV:C activity is unpredictable.

7 DISCUSSION

The use of locally collected pooled normal plasma as the reference point for the International Unit has been adopted for the quantitation of most coagulation factors (FII, FVII, FVIII, FIX, FX, XIII) and coagulation inhibitors (Antithrombin, Protein C, Protein S). The same approach has been adopted in the current study for the calibration of the Proposed WHO 1st IS FV Plasma.

By far the majority of laboratories rely on the estimation of clotting activity (FV:C) for FV quantitation with only a small number of specialist laboratories measuring FV by other means (antigen, chromogenic methods). For this reason the present calibration was restricted to Factor V clotting activity (FV:C). Within this group most laboratories use thromboplastin-based rather than APTT-based methods and this is reflected in the present study where only two out of 23 estimates of FV:C used APTT-based methods.

Factor V is recognised as a labile coagulation factor and can suffer loss of activity on storage in the liquid state or through cycles of freeze/thawing. For this reason the estimates of FV:C in the Proposed WHO 1st IS measured relative to fresh or frozen local pools were tabled separately (Table 2a). Loss of FV:C during freeze/thawing of the local pools would be expected to cause an apparent increase in the FV:C level in the Proposed WHO 1st IS when measured relative to the frozen pools in comparison to estimates relative to fresh pools. There is some evidence that this might be the case, in the present study, since the estimates for FV:C, relative to the frozen pools (mean 0.80 unit/ml), are significantly higher (p=0.047) than the estimates relative to the fresh pools (mean 0.74 unit/ml) (Table 2a). This is consistent with the finding of a higher mean FV:C concentration in the fresh local pools (Table 4). In agreement with the precedent set for the calibration of another labile factor, FVIII:C, it is proposed that the calibration of the Proposed WHO 1st IS FV Plasma be based on the mean of the 13 estimates relative only to the fresh local pools (0.74 IU/ampoule). Considering the large number of donors used to make the fresh pools (512) this should allow the IU to approximate to the population mean. This approach is supported by the observation that the fresh pools used in the present study were prepared from a larger number of donors than the pools used in the calibration of the WHO 4th IS and 5th IS FVIII/VWF Plasmas (360 and 240 donors respectively) (9,10).

Within laboratory (intra-laboratory) variability for the estimates of FV:C relative to the fresh local pools was low with a maximum GCV of 10.6% and the inter-laboratory variability (GCV 7.6%, n=13) was markedly lower than that found for estimates of FVIII:C relative to fresh pools during the calibration of the WHO 4th IS and 5th IS FVIII/VWF Plasma (GCV 18.3 % and 12.6 % respectively) (9,10). This might be an indication that the FV clotting assay is overall more robust than assays of FVIII:C. However, the low inter-laboratory variability found for FV:C in the present study is not reflected in the results of external quality assurance surveys where laboratory results differed by up to 20% when different commercial reference plasmas were used (3). Use of a common standard or common route of calibration (vs the WHO IS) should help to overcome such discrepancies and this is demonstrated in the present study by the extremely low inter-laboratory variability (GCV 3.55%) found for 23 estimates of FV:C in the SSC Lot #3 when calculated relative to the proposed WHO 1st IS (Table 3). Moreover, the good agreement between the two estimates obtained using the APTT-based methods with the 21 estimates using the thromboplastin-based methods indicates that the proposed WHO 1st IS is suitable for estimates with both methods. Considering that the Proposed WHO 1st IS is primarily intended to be used for the calibration of secondary reference plasmas (such as the SSC/ISTH Secondary Coagulation Standard Lot #3), rather than the direct estimation of FV:C in patient plasmas, this comparison also serves to address the issue of commutability since it demonstrates that the Proposed WHO 1st IS is valid for the estimation (and calibration) of FV:C in such secondary reference plasmas using many different combinations of reagents and instruments.

Estimation of FV activity using an “in house” chromogenic method in one laboratory, relative to a frozen local pool, produced a potency of 0.80 unit/ampoule which agreed with the mean estimate for FV:C by clotting assays (0.80 unit/ampoule, n=9) relative to the frozen plasma pools. This is an encouraging result for the new assay method which may offer an alternative approach to FV estimation in the future.

Estimates of FV antigen from 3 laboratories gave a higher mean value (0.84 unit/ampoule) compared to the overall mean estimate for FV clotting activity relative to the fresh local pools (0.74 unit/ampoule). However, considering that the mean antigen value is relative to only 3 local plasma pools it is more appropriate to compare antigen and clotting results from only the three laboratories which performed both assay methods on the same pools (numbers 1,8,11). This provides mean values of 0.78 unit/ampoule for FV:C and 0.84 unit/ampoule for FV:antigen and an activity/antigen ratio of 0.93 which is consistent with little degradation of FV during processing of the freeze-dried standard. Factor V activity may be more resistant to degradation than FVIII activity since the activity/antigen ratio for the current WHO 5th IS FVIII/VWF Plasma (02/150) was 0.72 (10).

The predictions from the accelerated degradation study indicate that the Proposed WHO 1st IS will lose less than 0.01 % FV:C activity per year when stored at -20 ^{o}C. This prediction is similar to the loss of 0.007% per year found for another labile coagulation factor, FVIII:C, in the WHO 5th IS FVIII Plasma (10). This loss indicates that the Proposed WHO 1st IS is extremely stable at the bulk storage temperature and suitable for prolonged use. The predictions of loss for storage at +20 ^{o}C ranged from 6 - 14 % per year; this result supports the routine shipment of standard at ambient temperature.

8 PROPOSAL

It is proposed that the WHO 1st IS Factor V Plasma (03/116) be assigned a value based on the estimates calculated relative to the fresh normal plasma pools of **0.74 IU Factor V:C per ampoule.**

9 RESPONSES FROM PARTICIPANTS

Responses have been obtained from all of the 22 participants. All responses have indicated agreement with the proposal to assign a mean value based on the estimates calculated relative to the fresh normal plasma pools of **0.74 IU Factor V:C per ampoule.**

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Table 1 Details of methodology for Factor V estimation

Method	Lab No	Thromboplastin Reagent	Substrate Plasma	Instrument
Clotting	1	Recombiplastin	IL Hemosil	Electra 1600 C
	2	Neoplastine CI plus	Stago	STA Compact
Thromboplastin-based	3	Dade Innovin	Cryocheck	Sysmex CA7000
	4	Simplastin HTF	Cryocheck	MDA-180
	5	Thromborel S	Dade Behring	ACL 9000
	7A	PT-Fibrinogen HS	IL Hemosil	ACL Futura
	7B	Recombiplastin	IL Hemosil	ACL Futura
	8	Neoplastine CI plus	Stago	STA-R
	9	Thromborel S	Trinity Biotech	STA compact
	10	PT-Fibrinogen HS	Technoclone	ACL 300R
	11	Dade Innovin	Dade Behring	Sysmex CA 6000
	12	Recombiplastin	Cryocheck	STA-R
	13	Thromborel S	Technoclone	KC10
	14	Thromborel S	Dade Behring	Behring BCT
	15	Neoplastine CI plus	Stago	STA-R
	16	Neoplastine CI plus	Stago	STA-R
	17A	Recombiplastin	Cryocheck	STA-R
	17B	Manchester	Helena	STA-R
	19	Reagent	Biomerieux	Electra 1800
	21	Recombiplastin	Diagen	ACL Futura
	23	Dade Innovin	IL Hemosil	ACL 9000
		PT-Fibrinogen HS		
Method	**Lab No**	**APTT Reagent**	**Substrate Plasma**	**Instrument**
Clotting	6	Platelin LS	Biomerieux	MDA II
	18	Dade Behring	Dade Behring	Dade Behring BCS
APTT-based				
Method	**Lab No**	**Assay type**	**Kit or "in house"**	**Reagents**
Chromogenic	20	microplate	"in house"	"in house"
Method	**Lab No**	**Assay type**	**Kit or "in house"**	**Antibodies**
Antigen	1	ELISA	"in house"	sheep anti-human
	8	ELISA	Zymutest FV	mono + polyclonal
	11	ELISA	"in house"	sheep anti-human

Table 2 Estimates for Factor V (units/ampoule) in the Proposed WHO 1st IS Factor V Plasma (03/116) relative to the normal plasma pools

a) Factor V Clotting Activity

	Fresh pools			Frozen pools		
Laboratory	**n**	**Mean**	**GCV%**	**n**	**Mean**	**GCV%**
1	4	0.79	5.54			
2	4	0.78	10.59			
3				4	0.71	9.33
4	4	0.62	2.31			
5	4	0.73	5.35			
6*				4	0.86	16.58
7a	4	0.77	4.18			
7b	4	0.76	2.03			
8	4	0.74	7.22			
9	4	0.79	6.62			
10				2	0.80	2.03
11				4	0.80	1.42
12				4	0.69	0.86
13				4	0.94	17.97
14	4	0.74	3.27			
15	4	0.81	7.85			
16				4	0.76	2.52
17a				4	0.84	2.33
17b				4	0.85	1.57
18**				*4*	*0.83*	*0.74*
19	4	0.72	4.44			
21	4	0.75	8.27			
23	4	0.68	7.41			
Mean estimates	**13**	**0.74**	**7.63**	**9**	**0.80**	**10.14**
Overall mean	**n = 22 mean 0.77 GCV 9.43%**					

All estimates were obtained using thromboplastin-based methods except for laboratories 6* and 18** which used APTT-based methods. The result from Laboratory 18** was not included in the combination since the normal pool was lyophilised.

b) Factor V Antigen

	Frozen pools		
Laboratory	**n**	**Mean**	**GCV%**
1	1	0.89	.
8	4	0.78	15.03
11	4	0.86	7.69
Overall mean estimate	**3**	**0.84**	**7.08**

Table 3 Estimates for Factor V clotting activity in the SSC/ISTH Secondary Coagulation Standard Lot #3 (coded B) expressed relative to the Proposed WHO 1st IS Factor V Plasma (coded A) using an assigned value of 100%

	B vs A		
Laboratory	**n**	**Mean**	**GCV%**
1	4	117	3.36
2	4	118	2.51
3	4	122	1.31
4	4	118	2.71
5	4	113	3.65
6*	4	116	16.14
7a	4	110	2.70
7b	4	117	1.74
8	4	118	1.31
9	4	119	3.03
10	4	124	2.57
11	4	124	1.47
12	4	120	1.55
13	4	111	10.05
14	4	122	2.40
15	4	117	2.45
16	4	119	2.06
17a	4	121	2.66
17b	4	114	2.77
18*	4	118	1.04
19	4	122	4.52
21	4	118	2.11
23	4	110	1.16
Overall mean estimate	**23**	**118**	**3.55**

All estimates were obtained using thromboplastin-based methods except for laboratories 6* and 18* which used APTT-based methods.

Table 4 Estimates for Factor V clotting activity (IU/ml) in the local normal plasma pools.

Laboratory	**Fresh pools**	**Frozen pools**
1	0.94	
2	0.95	
3		1.04
4	1.19	
5	1.01	
6*		0.86
7a	0.96	
7b	0.97	
8	1.00	
9	0.94	
10		0.93
11		0.93
12		1.07
13		0.79
14	1.00	
15	0.91	
16		0.97
17a		0.88
17b		0.87
18**		*0.89*
19	1.03	
21	0.99	
23	1.09	
Mean estimates	**n=13 mean 1.00 GCV 7.35%**	**n=9 mean 0.92 GCV 10.07%**

Estimates were calculated relative to the Proposed WHO 1st IS FV Plasma using a mean value of 0.74 IU per ml. All laboratories used thromboplastin-based methods except for 6* and 18** which used APTT-based methods. The local normal pool for laboratory 18** was lyophilised and the value has not been included in the combined estimates

Table 5 Residual mean potencies of ampoules stored at elevated temperatures as % of ampoules stored at -20 °C

Lab No	Storage period (years)	Residual mean potencies (% of -20 °C ampoules)			
		4 °C	20 °C	37 °C	45 °C
4	0.31	*****	*****	59	33
	1.85	98	83	*****	*****
15	0.31	*****	*****	63	40
	1.85	100	89	*****	*****
21	0.25	*****	*****	53	31
	1.79	94	76	*****	*****

Relative potencies are the means of four individual estimates

Table 6 Predicted % loss per year for ampoules stored at various temperatures

Lab No	Storage temperature		
	-20 °C	+4 °C	+20 °C
4	0.008 %	0.766 %	9.930 %
15	0.003 %	0.366 %	6.062 %
21	0.005 %	0.841 %	14.481 %

Figure 1 FV:C in the proposed 1st IS relative to the normal pools

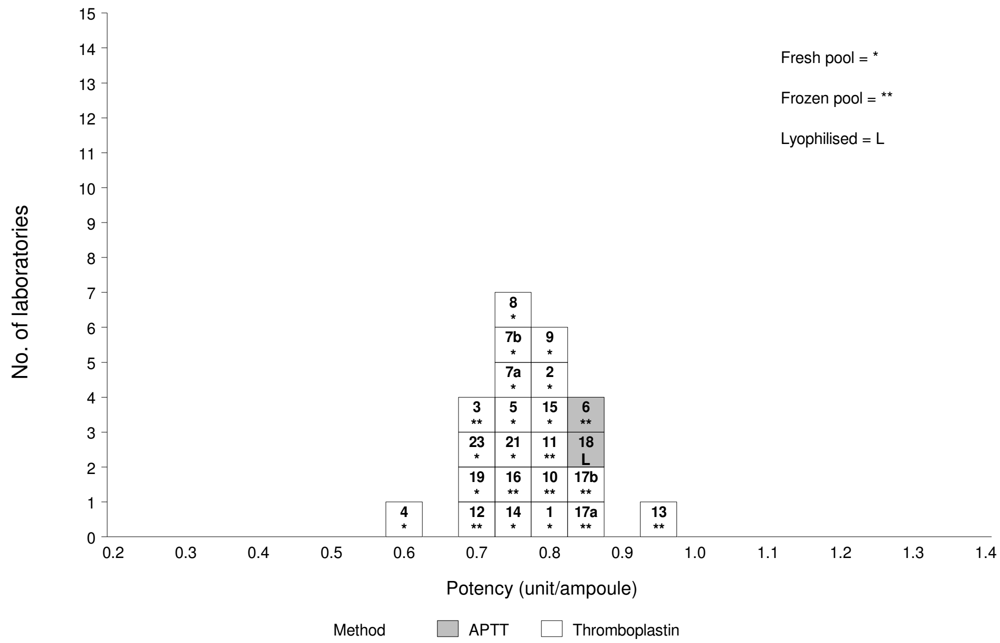


Figure 2 FV:C in the SSC/ISTH Secondary Coagulation Standard Lot#3 relative to 1st IS

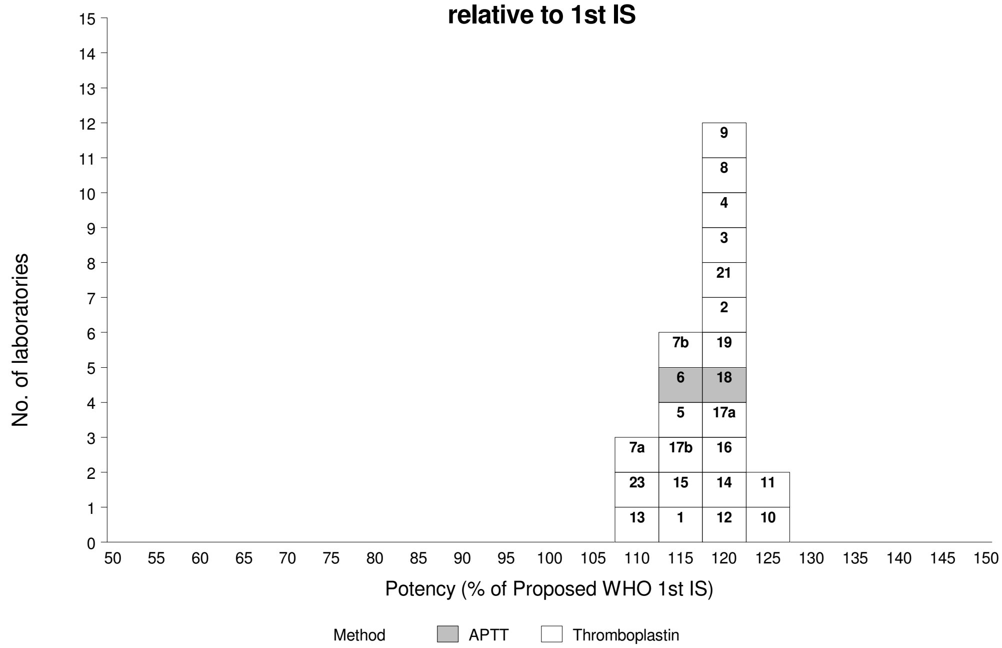
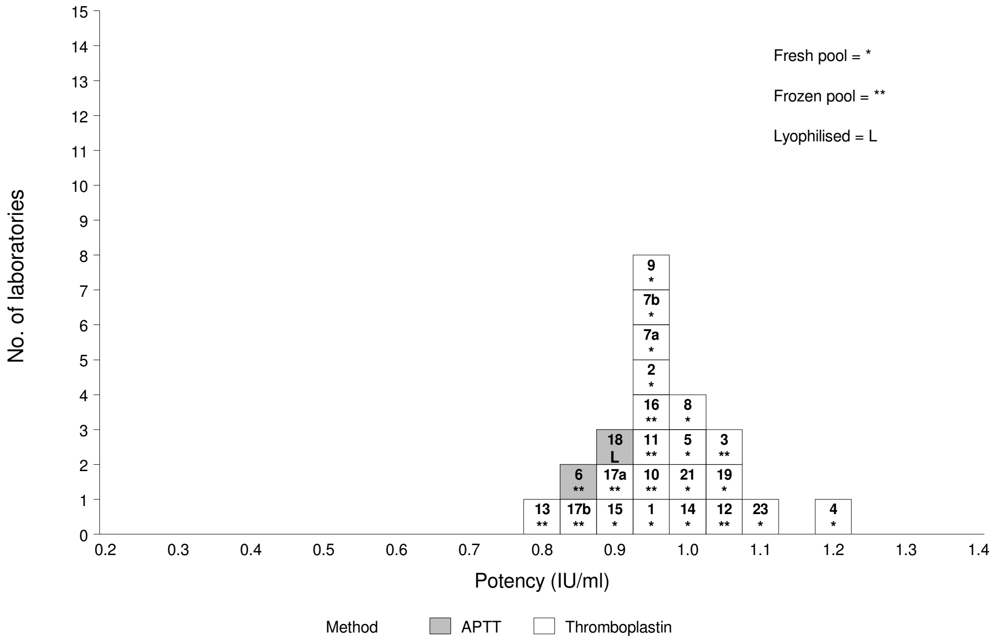


Figure 3 Local normal pools relative to 1st IS



APPENDIX 1 COLLABORATIVE STUDY PROTOCOL

1st INTERNATIONAL STANDARD FACTOR V PLASMA (03/116)

PROTOCOL FOR THE COLLABORATIVE STUDY

1 SAMPLES INCLUDED IN THE ASSAYS

A - Proposed 1st IS Factor V Plasma (03/116) - provided
B - SSC/ISTH Secondary Coagulation Standard Lot #3 - provided
N_1, N_2 - fresh normal plasma pools prepared locally (see section 5)

2 STORAGE AND RECONSTITUTION OF SAMPLES A AND B

Store the unopened ampoules and vials of A and B at -20^{o}C or below. Allow the ampoules and vials to warm to room temperature before reconstitution. Tap gently to ensure that all of the contents are in the lower part of the ampoules and vials. Reconstitute by adding 1.0 ml of distilled water. Dissolve the contents with gentle agitation at room temperature. When reconstitution is complete transfer the entire contents to stoppered plastic tubes and store at 4^{o}C during the assay period.

3 GENERAL PLAN OF THE STUDY

You are requested to carry out 4 assays by each method using fresh ampoules and vials for each assay. Only 4 ampoules/vials of A and B are provided (plus 1 spare of each) and it will therefore be necessary to carry out estimates of FV:C and FV:antigen on the same ampoules and vials (where applicable).

Estimates of FV:C must be carried out on freshly reconstituted ampoules/vials and fresh normal plasma pools (N_1, N_2) whereas estimates of FV:antigen (where applicable) may be carried out on frozen aliquots.

The 4 assays should be spread over 2 separate days/sessions as follows:

Assay session	Normal pool	Ampoules of A and B	Assay number
Day 1	N_1	1	1
		2	2
Day 2	N_2	3	3
		4	4

If you are unable to prepare the two fresh local pools (N_1, N_2) you may alternatively include a single local frozen plasma pool in all 4 assays.

4 ASSAY DESIGN

All three preparations (A, B, N) are included in each of the 4 assays. A minimum of 3 dilutions of each preparation should be tested, in replicate, within each assay. Please follow a balanced assay design such as the optimal 18-place assay described below. In the following design, each letter refers to a separate set of three or more dilutions and A, A' and B, B' etc. refer to fresh sets of dilutions (replicates) made from the same ampoule.

Design for 18-place assay

Assay 1	A	B	N_1	N_1'	B'	A'
Assay 2	B	N_1	A	A'	N_1'	B'
Assay 3	B	A	N_2	N_2'	A'	B'
Assay 4	N_2	B	A	A'	B'	N_2'

5 COLLECTION OF FRESH NORMAL PLASMA

Collect fresh normal plasma on two separate days to prepare pools N_1 and N_2. The method of collection for the fresh normal plasma is an important part of the study and should be standardised as far as possible according to the following protocol.

Donors Normal healthy volunteers, excluding pregnant women, women taking oral contraceptives and known carriers of the FV Leiden or HR2 alleles. (There is no requirement to screen your donors for these alleles). Take blood from as many different individuals as possible, on two separate days. If possible, use a minimum of 8 different donors for each pool; if this is not possible, some of the same individuals can be used again, but the aim is to have as many different donors as possible from each laboratory.

Anticoagulant 0.109 mol/L tri-sodium citrate or a mixture of tri-sodium citrate and citric acid with a total citrate concentration of 0.109 mol/L. Add 9 volumes of blood to 1 volume of anticoagulant.

Centrifugation Blood should be centrifuged at 4^oC as soon as possible after collection either at 50,000 *g* for 5 minutes or at 2,000 *g* for 20 minutes.

Storage Keep the plasma pool in a plastic stoppered tube at 4^oC during the assay session. Freeze aliquots of each pool (N_1, N_2) for subsequent assays of FV:antigen where applicable.

6 RESULTS

Please return your raw assay data on the enclosed results sheets to allow analysis at NIBSC. Please ensure that your results are presented as true raw data (eg. clotting time, optical density) rather than as % or units relative to an in house standard. You are also invited to calculate your own estimates for A and B relative to N_1 and N_2 if you wish (see enclosed results sheet).

Please return the results sheets and questionnaire to:

Dr A R Hubbard, Division of Haematology, NIBSC, Blanche Lane, South Mimms, Potters Bar, Herts. EN6 3QG United Kingdom
Fax: +44 (0) 1707 646730 E-mail: thubbard@nibsc.ac.uk

APPENDIX 2 LIST OF PARTICIPANTS

Dr A Hunfeld
Paul-Ehrlich-Institut
Langen Germany

Mrs J M Rentenaar
Sanquin Blood Supply Foundation
Amsterdam The Netherlands

Dr R Bader
A Bianchi Bonomi H&T Center
Milano Italy

Dr S Kitchen/Ms A Bowyer
Royal Hallamshire Hospital
Sheffield UK

Dr K Trumbull
Instrumentation Laboratory (QA)
Lexington MA USA

Dr M Shima
Nara Medical University
Nara Japan

Prof C Lee/Ms A Riddell
Royal Free Hospital
London UK

Mme C Caron
Hôpital Cardiologique - CHU
Lille France

Dr H Metzner
ZLB Behring
Marburg Germany

Mr F Nicham
Diagnostica Stago (R&D)
Gennevilliers France

Dr J Weinberger
Octapharma Pharmazeutika
Vienna Austria

Dr B Kerbl
Technoclone GmbH
Vienna Austria

Dr A Hubbard/Ms L J Weller
NIBSC
Potters Bar UK

Mme S Berrier
Diagnostica Stago (QC)
Franconville France

Dr R Veinotte/Dr S Duff
Precision Biologic Inc.
Dartmouth, NS Canada

Dr S Rosen
Rossix
Molndal Sweden

Dr C Loh
TGA Laboratories
Symonston Australia

Prof P Giangrande/Mr K Sukhu
Haemophilia Centre & Thrombosis Unit
Oxford UK

Prof J V Lloyd/Ms E Duncan
Inst. of Medical & Veterinary Science
Adelaide Australia

Dr A Tosetto / Dr A Cappelletti
Ospedale San Bortolo
Vicenza Italy

Dr J C J Eikenboom
University Medical Center
Leiden The Netherlands

Ms D Harz
Instrumentation Laboratory (QC)
Orangeburg NY USA

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