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**Report on the Factor V:C Calibration of the
Proposed WHO 1st IS Factor V Plasma (03/116) and the
SSC/ISTH Secondary Coagulation Standard Lot #3**

On behalf of the SSC Subcommittee on Factor VIII and Factor IX of the ISTH

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**NOTE ON ESTABLISHMENT OF THE WHO 1st IS FACTOR V PLASMA
(03/116)**

The preparation coded 03/116 was formally established in October 2005 by the Expert Committee on Biological Standardisation of the World Health Organisation as the WHO 1st International Standard, Factor V, Plasma with an assigned value of 0.74 IU Factor V:C per ampoule. The standard is available from the National Institute for Biological Standards and Control, Potters Bar, UK.
(www.nibsc.ac.uk)

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). A secondary objective of the study was the calibration of the SSC/ISTH Secondary Coagulation Standard Lot #3 (SSC Lot #3) relative to the Proposed WHO 1st IS.

Calibration of the Proposed WHO 1st IS (coded A) 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 IU/ampoule, mean 0.74 IU/ampoule) and 9 estimates relative to frozen pools (range 0.69 - 0.94 IU/ampoule, mean 0.80 IU/ampoule). Although there was no significant difference between the estimates calculated relative to the fresh and frozen pools the higher mean value for estimates relative to the frozen pools could indicate that some degradation of FV had occurred during freeze/thawing of the 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.

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Calibration of the SSC Lot #3 (coded B) was based on clotting assays relative to the Proposed WHO 1st IS using an interim value of 100%. The assigned mean value for SSC Lot #3 can be converted to IU/ml when the final assigned value for the Proposed WHO 1st IS has been agreed. Twenty three estimates were obtained comprising 21 using thromboplastin-based methods and 2 using APTT-based methods. There was very good agreement between the 23 estimates for FV:C which ranged from 110 - 124% with inter-laboratory variability (GCV) of 3.55% and an overall mean estimate of 118%. It is proposed that the SSC Lot #3 be assigned a value equivalent to 118% of the agreed value assigned to the Proposed WHO 1st IS.

PROPOSALS

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

2 It is proposed that the SSC/ISTH Secondary Coagulation Standard Lot #3 be assigned a value of 118% of the value assigned to the WHO 1st IS Factor V Plasma (03/116). This will be equivalent to 0.87 IU/ml if the above proposal is adopted.

INTRODUCTION AND OBJECTIVES OF THE STUDY

Estimation of Factor V clotting activity (FV:C) in plasma is performed for several reasons. These include the diagnosis of rare congenital bleeding disorders such as FV deficiency or combined FV/FVIII deficiency, the confirmation of liver disease as a cause of a prolonged PTT result and more recently in the quality control of virus-inactivated fresh frozen human plasma. 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. The primary objective of this study was 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.

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 IU per ml. This approach allows the IU to approximate to the population mean value for FV:C and so facilitates the definition and understanding of the normal and pathological ranges.

A secondary objective of the study was the calibration of the SSC/ISTH Secondary Coagulation Standard Lot #3 (SSC Lot#3). This standard is available to manufacturers of commercial calibrant plasmas for the assignment of potency. SSC Lot #3 was calibrated relative to the Proposed WHO 1st IS using an interim value of 100% for the Proposed WHO 1st IS. A value in IU can easily be

calculated for the SSC Lot #3 once the final assigned potency on the Proposed WHO 1st IS has been agreed.

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

MATERIALS

1. Candidate WHO 1st International Standard Factor V, Plasma (03/116)

The Proposed WHO 1st IS was prepared from a plasma pool derived from 26 normal healthy donors (UK Blood Service). Blood was collected using conventional venepuncture, centrifuged twice and the plasma stored at -70 °C until the day of ampoule filling. 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 minipools and found negative for the presence of HCV RNA using a PCR technique. During filling each glass ampoule received 1 ml of the pool, followed by freeze-drying and secondary desiccation according to the requirements for International Biological Standards (1). Details of ampoule filling are as follows: mean fill weight 1.0052 g (range 1.0038 - 1.0066 g); coefficient of variation of 62 fill checkweights 0.05 %; mean dry weight 0.0840 g; mean residual moisture 0.152 %.

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 plasma, freeze-dried.

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)

N₁, N₂ Normal plasma pools collected locally (see Appendix 1 for instructions on the method of preparation).

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.

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.

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.

STATISTICAL ANALYSIS

Wherever possible assays were analysed as parallel line bioassays (2), 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) (3). 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 (4).

RESULTS

Assay data and validity

A total of 109 assays were returned from the 22 participants comprising 88 estimates of Factor V clotting (thromboplastin-based), 8 assays of Factor V clotting (APTT-based), 4 assays of Factor V by chromogenic method (1 laboratory) and 9 assays of Factor V antigen (3 laboratories). Significant deviations from parallelism, at the 1% level, were found for 6 out of 109 assays and significant deviations from linearity, at the 1% level, were found for 12 out of the total of 327 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.

1 PROPOSED WHO 1st IS (A) vs NORMAL POOLS (N₁, N₂)

1a) FACTOR V CLOTTING

Mean estimates from the individual laboratories ranged from 0.62 to 0.94 IU/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 IU/ampoule with an overall mean of 0.74 IU/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 IU/ampoule with an overall mean of 0.80 IU/ampoule and inter-laboratory variability (GCV) of 10.14 %. Estimates relative to the fresh pools were not significantly different to estimates relative to the frozen pools at the 1% level (t-test p=0.047). Combination of all 22 estimates gave a mean of 0.77 IU/ampoule and inter-laboratory variability (GCV) of 9.43 %.

1b) FACTOR V 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 IU/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 IU/ampoule with inter-laboratory variability (GCV) of 7.08% (Table 2b).

2 SSC/ISTH SECONDARY COAGULATION STANDARD LOT #3 (B) vs PROPOSED WHO 1st IS FACTOR V PLASMA (A)

2a) FACTOR V CLOTTING

Twenty three estimates were obtained and these ranged from 110% to 124% of the Proposed WHO 1st IS (Table 3a 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%.

2b) FACTOR V BY OTHER METHODS

One laboratory (number 20) used an “in house” chromogenic method to estimate FV activity. The mean estimate from 4 assays was 103% of the Proposed 1st IS with intra-laboratory variability of 7.26%.

Estimates for FV antigen, from three laboratories, were very close at 116, 118 and 117% of the Proposed WHO 1st IS with an overall mean of 117% and inter-laboratory variability of 0.94% (Table 3b).

3 VARIABILITY OF THE LOCAL NORMAL PLASMA POOLS

Estimates of the FV:C content in the different local normal pools have been 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 no significant difference in FV:C content between the fresh and frozen pools at the 1% level (t-test p=0.046).

DISCUSSION

Calibration of the Proposed WHO 1st IS Factor V Plasma

The use of locally collected pooled normal plasma as the reference point for the International Unit has been used for the quantitation of most coagulation factors (FII, FVII, FVIII, FIX, FX, XIII) and 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 minority of 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 cause an apparent increase in the FV:C level in the Proposed WHO 1st IS for estimates measured relative to the frozen pools when compared to estimates relative to fresh pools. There is some evidence that this might be the case, in the present study, since the mean result for FV:C, relative to the frozen pools (0.80 IU/ml), is higher than the mean result relative to the fresh pools (0.74 IU/ml) (Table 2a) hence 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 calibration of the WHO 4th IS and 5th IS FVIII/VWF Plasmas were prepared from less donors (360 and 240 respectively) (5,6).

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) (5,6). 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 can differ by up to 20% when different reference plasmas are used. Use of a common standard or common route of calibration (vs the WHO IS) should help to overcome such discrepancies and this is supported by the extremely low inter-laboratory (GCV 3.55%) found for 23 estimates of FV:C in the SSC Lot #3 relative to the proposed WHO 1st IS (Table 3).

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 IU/ampoule which agreed with the mean estimate for FV:C by clotting assays (0.80 IU/ampoule, n=9) relative to the frozen plasma pools.

Estimates of FV antigen from 3 laboratories gave a higher mean value (0.84 IU/ampoule) compared to the overall mean estimate for FV clotting activity relative to the fresh local pools (0.74 IU/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 IU/ampoule for FV:C and 0.84 IU/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. Interestingly a similar activity/antigen ratio was found for FV in the SSC Lot #3 (0.95). This indicates that the different levels of FV:C in the proposed WHO 1st IS and SSC Lot #3 are simply caused by different amounts of FV protein rather than different degrees of degradation during processing. Factor V activity may be more resistant to degradation than FVIII activity since the activity/antigen ratio was 0.72 for the current WHO 5th IS FVIII/VWF Plasma (02/150) (6).

Calibration of the SSC/ISTH Secondary Coagulation Standard Lot #3

Local plasma pools were not involved in the calibration of the SSC/ISTH Secondary Coagulation Standard Lot #3 which was carried out by direct comparison with the Proposed WHO 1st IS. This allowed the inclusion of results from all 23 laboratories which assayed FV clotting activity. Intra-laboratory variability of estimates was low with 20/23 laboratories having a GCV below 4%. Inter-laboratory variability was also low with a GCV (n=23) of only 3.55% as might be expected since all laboratories compared the same materials. It is proposed that an interim mean value of 118% of the Proposed WHO 1st IS be assigned. This will be equivalent to a value of 0.87 IU/ml if the value of 0.74 IU/ampoule is accepted for the Proposed WHO 1st IS.

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Table 1 Details of methodology for Factor V estimation

Method	Lab No	Thromboplastin Reagent	Substrate Plasma	Instrument
Clotting Thromboplastin-based	1	Recombiplastin	IL Hemosil	Electra 1600 C
	2	Neoplastine CI plus	Stago	STA Compact
	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 Reagent	Helena	STA-R
	19	Recombiplastin	Biomerieux	Electra 1800
	21	Dade Innovin	Diagen	ACL Futura
23	PT-Fibrinogen HS	IL Hemosil	ACL 9000	
Method	Lab No	APTT Reagent	Substrate Plasma	Instrument
Clotting APTT-based	6	Platelin LS	Biomerieux	MDA II
	18	Dade Behring	Dade Behring	Dade Behring BCS
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 (IU/ampoule) in the Proposed WHO 1st IS Factor V Plasma (03/116) relative to the normal plasma pools

a) Factor V Clotting Activity

Laboratory	Fresh pools			Frozen pools		
	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

Laboratory	Frozen pools		
	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 (%) in the SSC/ISTH Secondary Coagulation Standard Lot #3 (coded B) relative to the Proposed WHO 1st IS Factor V Plasma (coded A) using an assigned value of 100%

a) Factor V Clotting Activity

Laboratory	B vs A		
	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.

b) Factor V Antigen

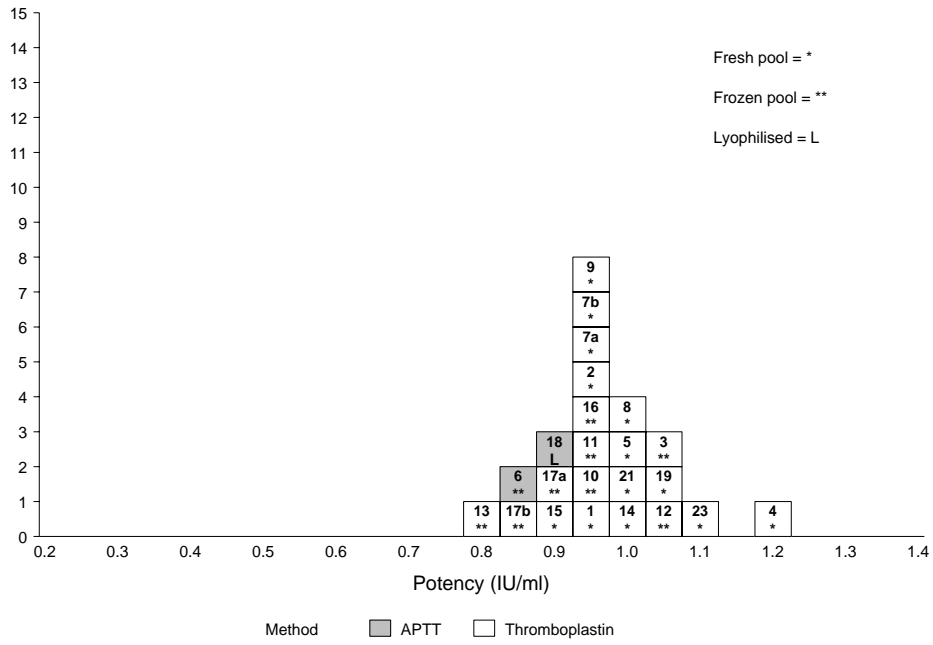
Laboratory	B vs A		
	n	Mean	GCV%
1	1	116	.
8	4	118	4.96
11	4	117	4.64
Overall mean estimate	3	117	0.94

Table 4 Estimates for Factor V (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

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
N₁, N₂ - 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°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°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 (N₁, N₂) 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 (N₁, N₂) 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 N₁ and N₂. 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°C 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°C during the assay session. Freeze aliquots of each pool (N₁, N₂) 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 N₁ and N₂ if you wish (see enclosed results sheet). Please return the results sheets and questionnaire to:

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APPENDIX 2 LIST OF PARTICIPANTS

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ADDENDUM TO REPORT

PROPOSED WHO 1st IS FACTOR V PLASMA (03/116)

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 °C) relative to ampoules stored at the bulk storage temperature of -20 °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 (Kirkwood and Tydeman, 1984).

Three laboratories participated in the study and their results are presented in Table 1. Estimates of predicted loss per year for storage at -20, +4 and +20 °C are presented in Table 2.

Table 1 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 2 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 %

CONCLUSIONS

Results from all three laboratories are consistent with a loss of FV:C below 0.01 % per year for ampoules at the bulk storage temperature of -20 °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 °C ranged from 6 to 14 % per year and this supports the shipment of ampoules at ambient temperature.

REFERENCE

Kirkwood TBL & Tydeman (1984) Design and analysis of accelerated degradation tests for the stability of biological standards II. A flexible computer program for data analysis. *J Biol Standardisation* 12; 207-14