World Health Organization

WHO/BS/2011.2171
ENGLISH ONLY

EXPERT COMMITTEE ON BIOLOGICAL STANDARDIZATION
Geneva, 17 to 21 October 2011

Assignment of a value for von Willebrand factor propeptide to the WHO 6th IS Factor VIII/von Willebrand factor, Plasma (07/316)

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Summary

Background and aim:

The ratio of VWF propeptide/VWF:antigen (VWFpp/VWF:Ag) can be used to identify bleeding disorders associated with decreased half-life of VWF in the circulation (eg. type 1 von Willebrand disease with increased clearance, acquired von Willebrand syndrome). Although there is an agreed international unitage (IU) for VWF:Ag, there is no agreed IU for VWFpp. This collaborative study has been undertaken to assess the inter-laboratory variability of VWFpp (and VWF:Ag) estimates and to calibrate the WHO 6th IS FVIII/VWF Plasma (07/316) with an agreed unitage for VWFpp.

Methods and Results:

An international multi-centre collaborative study involving 13 laboratories was undertaken to test two freeze-dried plasma samples, the WHO 6th IS FVIII/VWF Plasma (WHO IS Plasma) and the SSC/ISTH Secondary Coagulation Standard Lot #3 (SSC Lot #3) for VWFpp and VWF:Ag relative to the participants' local reference materials.

The inter-laboratory variability for VWFpp estimates was surprisingly low for both freeze-dried plasma samples (GCVs 8.1%, 8.8%) considering that different local reference materials were used in each laboratory. This variability was reduced even further (GCV 3.0%) when estimates were calculated relative to the same plasma sample (SSC Lot #3 vs WHO IS Plasma) indicating that the different methodologies for VWFpp estimation are generally in good agreement and that the availability of an international reference material/unitage could further improve agreement between laboratories. The inter-laboratory variability for VWFpp estimates was sufficiently low to allow the assignment of consensus mean values to both the WHO IS Plasma and the SSC Lot #3 of 1.03 IU/ampoule and 1.01 IU/ml respectively.

The inter-laboratory variability for VWF:Ag estimates, relative to local reference materials (GCVs 12.2%, 12.0%), was larger than that found for VWFpp estimates despite the availability of the WHO IS Plasma for calibration of VWF:Ag. This larger variability is probably caused by inaccurate calibration of the local reference materials rather than different methodologies since the variability was greatly reduced (GCV 3.5%) when all laboratories compared the same plasma samples (SSC Lot #3 vs WHO IS Plasma).

This study indicates that improved agreement between laboratories in the estimation of the VWFpp/VWF:Ag ratio could be achieved through the establishment of a WHO IS with agreed unitage for VWFpp but there is also a need for better harmonisation in the calibration of local references for VWF:Ag.

Proposal:

It is proposed that the WHO 6th IS FVIII/VWF Plasma (07/316) be assigned a value of 1.03 IU/ampoule for von Willebrand factor propeptide.

Introduction and objectives of the study

Determination of the ratio for VWF propeptide/VWF:antigen (VWFpp/VWF:Ag) has diagnostic applications for conditions associated with decreased half-life of VWF in the circulation (eg. type 1 von Willebrand disease with increased clearance, acquired von Willebrand syndrome) (1,2). Although there is an agreed international unitage (IU) for VWF:Ag, currently assigned to the WHO 6th IS FVIII/VWF Plasma, there is no agreed IU for VWFpp. Laboratories therefore rely on local or "unofficial" reference preparations for VWFpp estimation with consequent potential for considerable inter-laboratory variability as well as problems of long-term continuity. The present study was undertaken with the following objectives:
- to assess the inter-laboratory variability of VWFpp (and VWF:Ag) estimates
- to assign a value for VWFpp to the WHO 6th IS FVIII/VWF Plasma (07/316).

Assignment of a value for VWFpp to the WHO 6th IS FVIII/VWF Plasma will provide long-term continuity for the VWFpp unit and should lead to improved agreement between laboratories by providing a traceable route for the calibration of all secondary working standards.

These objectives have been addressed in the present collaborative study through the testing of two lyophilized plasma samples, WHO 6th IS FVIII/VWF Plasma (WHO IS Plasma) and the SSC/ISTH Secondary Coagulation Standard Lot #3 (SSC Lot #3) relative to local reference preparations for VWFpp and VWF:Ag. This study has allowed the assessment of the inter-laboratory variability for both analytes and also given the opportunity to assign a value for VWFpp to the WHO 6th IS FVIII/VWF Plasma. This project was endorsed by WHO ECBS in October 2010.

The unitage for VWF propeptide

The test for VWFpp is already established in many laboratories and the majority of results are reported relative to local reference plasmas which have assigned values in units where 1 unit is equivalent to the VWFpp in 1 ml of pooled normal plasma. In order to minimize discontinuity of the unitage, when a value is assigned to the WHO IS Plasma, it was proposed that this concept should be continued. It was agreed that the WHO IS Plasma should be assigned the consensus mean value of estimates calculated relative to the local reference plasmas and, following the precedent for other standards in the haemostasis field, the assigned value will be labeled in International Units (IU) (3).

Samples included in the collaborative study

WHO 6th IS Factor VIII/von Willebrand factor, plasma (07/316) (WHO IS Plasma) – this is the current WHO International Plasma Standard for factor VIII:C, factor VIII:antigen, VWF:antigen, VWF:ristocetin cofactor and VWF:collagen binding. This standard was prepared in accordance with WHO recommendations (4) and was established in 2009. Full details of the collaborative study and the physical properties of the ampouled material are available in ECBS report WHO/BS/09.2116 (5).

SSC/ISTH Secondary Coagulation Standard Lot #3 (SSC Lot #3) – this secondary standard consists of a freeze-dried pooled normal plasma, calibrated for 20 analytes,

and is available to manufacturers for the value assignment of commercial plasma reference preparations.

Local in house reference for VWFpp – this was provided by the participants in the collaborative study and details are given in Appendix 1.

Local in house reference for VWF:Ag - this was provided by the participants in the collaborative study and details are given in Appendix 1.

Participants and Study Design

Samples were despatched in October 2010 to 13 laboratories (7 countries) comprising 8 clinical laboratories and 5 manufacturers (Appendix 2). Each laboratory has been assigned a confidential code number which does not relate to the list order in Appendix 2. Participants were requested to follow their routine methodology as far as possible within the specified assay design (Appendix 3). Each laboratory performed a total of 4 assays for VWFpp and 4 assays for VWF:Ag using fresh ampoules/vials of WHO IS Plasma and SSC Lot #3 and fresh samples of local in house references in each assay. It was requested that the 4 assays be performed over at least 2 different days with each assay following a balanced design including multiple dilutions of each test sample to allow the generation of a dose-response relationship for each preparation. Details of the methods used by the participants are listed in Appendix 1 together with the assigned values for the local reference preparations. All VWFpp methods were ELISA-based: 5 laboratories used reagents provided in the GTI Diagnostics kit; 5 laboratories used antibodies provided by Sanquin and 3 laboratories used in house methods and reagents. Twelve out of thirteen laboratories used ELISA methods for VWF:Ag: 5 laboratories used reagents provided in the GTI Diagnostics kit; 4 laboratories used antibodies purchased from Dako; 3 laboratories used in house methods and reagents. One laboratory used the Siemens turbidimetric method for VWF:Ag.

Local Reference Materials

VWFpp: 8 laboratories used frozen normal plasma pools as local references; 1 laboratory used a lyophilised, pooled normal plasma; 2 laboratories used a single GTI kit calibrator; 1 laboratory used a purified preparation of recombinant VWFpp and 1 laboratory used frozen aliquots of SSC Lot #3 (Appendix 1). Value assignments were in units per ml for all laboratories except for laboratories 1 (mg/ml) and 10 (nM); values in units were either arbitrarily assigned (1.0 u/ml) or were based on the value for VWF:Ag.

VWF:Ag: 7 laboratories used frozen normal plasma pools as local references; 1 laboratory used a lyophilised, pooled normal plasma; 2 laboratories used the WHO 1st IS VWF Concentrate; 2 laboratories used a single GTI kit calibrator; 1 laboratory used frozen aliquots of SSC Lot #3 (Appendix 1). Value assignments were in International Units (IU) per ml traceable to the WHO International Standards except for laboratory 10 where the assigned value was in ug/ml.

Note on laboratories using the GTI kit method: the 5 laboratories using the GTI kit routinely estimated VWFpp and VWF:Ag relative to the kit control samples rather than a local reference material. However, in the present study these laboratories altered their routine

procedure by including a "temporary" local reference material which consisted of a frozen normal plasma pool (labs 3, 12, 13) or one of the GTI kit calibrators (labs 2, 7). Three of these laboratories (labs 7, 12, 13) also estimated VWFpp and VWF:Ag in all preparations (including the local reference) relative to the GTI kit controls. In order that the results were most representative of routine procedure it was decided to include the results calculated by the participants relative to the GTI kit controls where possible (labs 7, 12, 13) rather than against the "temporary" local reference materials.

Statistical Analysis

All assays were analysed as multiple parallel line bioassays comparing response to log concentration (6). Linear and parallel response lines are required for this type of analysis and, if necessary, the responses were log transformed to achieve this. The parallelism of the assays was assessed by comparing the slopes of the dose-responses across the assays. For each VWFpp and VWF:Ag assay the estimates for the WHO IS Plasma and SSC Lot #3 were calculated relative to the local reference preparations. In addition, for each VWFpp and VWF:Ag assay, the estimates for the SSC Lot #3 were calculated relative to the WHO IS Plasma (using an arbitrary value of 1.0 units/ml for VWFpp and the assigned value of 1.00 IU/ml for VWF:Ag). Combined potency estimates for each laboratory were obtained by taking unweighted geometric means of results from all assays. Overall combined estimates were obtained by taking unweighted geometric means of the mean results from the different laboratories. Differences in potency estimates between laboratories (outlier detection) were assessed using Grubb's test (7). Intra- and inter-laboratory variability is expressed as the geometric coefficient of variation (GCV%) (8).

Results

General comments on the statistical analysis

A total of 52 assays were received for each method and all were valid for inclusion in the analysis. For two assays of VWFpp it was necessary to exclude the response from a single dilution in order to achieve linearity, otherwise all data was included. All laboratories reported VWFpp results as units/ml except for Labs 1 & 10 (mg/ml and nM respectively); the results from these 2 laboratories were not included in the overall mean value for estimates relative to local references. All laboratories reported VWF:Ag results as IU/ml except for laboratory 10 (ug/ml); these results were not included in the overall mean value for estimates relative to local references. There were no outlying results for VWFpp or VWF:Ag.

von Willebrand factor propeptide

Intra- and inter-laboratory variability of estimates. Estimates of intra-laboratory variability were based on the 4 independent assays returned by each participant and ranged from GCV of 1.0% to 17.0%. Variability was generally low with 34 out of 39 datasets with GCV below 10% and 21 out of 39 datasets with GCV below 5% (Tables 1 & 2). Inter-laboratory variability was very similar for the estimates of the WHO IS Plasma and the SSC Lot #3 relative to the local reference materials with GCVs of 8.1% and 8.8% respectively. This variability is low considering the use of different local reference materials in each

laboratory. As expected the inter-laboratory variability for estimates comparing the SSC Lot #3 relative to the WHO IS Plasma was considerably lower with a GCV of 3%.

VWFpp estimates relative to local reference materials. Mean laboratory estimates returned as units/ml for the WHO IS Plasma and the SSC Lot #3, relative to the local reference materials, both ranged from 0.92 to 1.21 units/ml with overall means of 1.03 units/ml (n=11) and 1.01 units per ml (n=11) respectively and there were no outlying results (Table 1, Figures 1 & 2). There were no significant differences between estimates obtained using the Sanquin reagents (n=5) and the GTI kit (n=5) for both the WHO IS Plasma (p=0.81) and SSC Lot #3 (p=0.64).

VWFpp estimates for SSC Lot #3 relative to WHO IS Plasma. Using an arbitrary assigned value of 1.00 units/ml for the WHO IS Plasma, the relative estimates for SSC Lot #3 ranged from 0.94 to 1.03 units/ml with an overall mean of 0.98 units/ml (Table 2, Figure 3). There was no significant difference (p=0.45) between estimates obtained using the Sanquin reagents (n=5) and the GTI kit (n=5).

von Willebrand factor antigen

Intra- and inter-laboratory variability of estimates. Estimates of intra-laboratory variability were based on the 4 independent assays returned by each participant and ranged from GCV of 0.6% to 23.9%. Variability was generally low with 34 out of 39 datasets with GCV below 10% and 22 out of 39 datasets with GCV below 5% (Tables 3 & 4). Inter-laboratory variability was very similar for the estimates of the WHO IS Plasma and the SSC Lot #3 relative to the local reference materials with GCVs of 12.2% and 12.0% respectively. As expected the inter-laboratory variability for estimates comparing the SSC Lot #3 relative to the WHO IS Plasma was considerably lower with a GCV of 3.5%.

VWF:Ag estimates relative to local reference materials. Mean laboratory estimates returned as IU/ml, relative to the local reference materials, ranged from 0.73 to 1.09 IU/ml with overall mean of 0.93 IU/ml (n=12) for the WHO IS Plasma and from 0.85 to 1.22 IU/ml with overall mean 1.05 IU/ml (n=12) for the SSC Lot #3. There were no outlying results (Table 3).

VWF:Ag estimates for SSC Lot #3 relative to WHO IS Plasma. Mean laboratory estimates ranged from 1.06 to 1.22 IU/ml with an overall mean of 1.13 IU/ml (Table 4).

Ratios of VWFpp/VWF:Ag

Ratios for the WHO IS Plasma and SSC Lot #3, relative to the local references, ranged from 0.844 to 1.346 and from 0.760 to 1.129 respectively with inter-laboratory variability (GCV) of 14.5 and 14.3% respectively (Table 5). Ratios for the SSC Lot #3, relative to the WHO IS Plasma ranged from 0.820 to 0.953 with inter-laboratory variability of 3.8%.

Discussion

The estimation of VWFpp currently relies on the use of local reference preparations which have been assigned arbitrary values in the absence of an agreed VWFpp unit. The main objectives of this study were to investigate, a) the inter-laboratory variability of VWFpp estimates and, b) the possibility of assigning a value for VWFpp to the WHO 6th IS FVIII/VWF Plasma (07/316). All of the data returned by the participants were statistically valid, however, the following conclusions have certain qualifications:
- two out of the 13 laboratories (labs 1 & 10) did not report VWFpp estimates in units/ml and therefore these laboratory estimates, relative to the local references, were not included in the overall combined results. However, the results from all laboratories were included in the comparison of the SSC Lot #3 relative to the WHO IS Plasma.
- in order to include the results which were most representative of routine testing it was decided to use the VWFpp estimates calculated by laboratories 7, 12 and 13, relative to the GTI kit controls, rather than to use estimates calculated relative to the "local reference materials" which were only introduced for this study.

Variability of VWFpp estimates

The inter-laboratory variability for estimates of the WHO IS Plasma (GCV 8.1%) and the SSC Lot #3 (GCV 8.8%), relative to the local reference materials, was low considering that different local reference materials, with arbitrary value assignments, were used in most laboratories. However, the inter-laboratory variability was further reduced considerably (GCV 3.0%) when all laboratories compared the same materials (SSC Lot #3 vs WHO IS Plasma). This is an indication that the different methodologies for VWFpp estimation are in good agreement and that an international reference material/unitage could lead to further improvement in agreement between laboratories.

Proposed value assignment for VWFpp

The objective of assigning a value for VWFpp to a reference material would be to provide a single traceable source for the calibration of all secondary (working) standards. Ideally this reference preparation should fulfil the requirements for homogeneity, longevity and stability and this could be achieved by the assignment of a value to the WHO 6th IS FVIII/VWF Plasma. The most obvious approach to value assignment for VWFpp would be to apply the consensus mean of estimates calculated relative to the local reference materials in units/ml, as given in Table 1, since this would be consistent with the convention currently followed by the majority of laboratories. The validity of the consensus mean approach is further supported by the low inter-laboratory variability (GCV) of 8.1% for estimates of the WHO IS Plasma relative to the local reference materials. It is therefore proposed that a VWFpp value of 1.03 IU/ampoule be assigned to the WHO 6th IS FVIII/VWF Plasma. Subject to acceptance of this assignment by WHO it will be then be valid to calibrate the secondary standard, SSC Lot #3, using the estimates relative to the WHO IS given in Table 2.

Estimates for VWF:Ag

The determination of VWF:Ag was included in the present study since the recognition of a reduced plasma half-life for VWF is based on the VWFpp/VWF:Ag ratio. Hence the variability of the ratio will be dependent on the variability of both VWFpp and VWF:Ag estimates. It is surprising to note that, despite the existence of the WHO IS Plasma with an agreed IU for VWF:Ag, estimates of VWF:Ag in both the WHO IS Plasma and the SSC Lot #3, relative to the local references, were more variable (GCVs 12.2 and 12.0% respectively)

than estimates of VWFpp (GCVs 8.1 and 8.8% respectively). This larger variability for VWF:Ag is probably caused by the inaccurate calibration of the local reference materials rather than from effects of different methodologies since the inter-laboratory variability for VWF:Ag when the same samples were compared (SSC Lot #3 vs WHO IS Plasma) was reduced greatly (GCV 3.5%).

Ratios of VWFpp/VWF:Ag

As expected the inter-laboratory variability for ratios of the WHO IS Plasma and SSC Lot #3, relative to the local reference materials (GCVs 14.5 and 14.3% respectively) exceeded the inter-laboratory variability of the individual VWFpp and VWF:Ag estimates. However, the inter-laboratory variability of the ratios was reduced greatly (GCV 3.8%) when the estimates were calculated relative to the same reference material (SSC Lot #3 vs WHO IS Plasma) and this indicates that the calibration of the local reference materials, rather than methodological differences, is probably the largest cause of variability.

Overall this study indicates that increased agreement between laboratories in the estimation of the VWFpp/VWF:Ag ratio may be achieved through the establishment of a WHO IS with agreed unitage for VWFpp but there is also a need for better harmonisation in the calibration of local references for VWF:Ag.

Proposal for value assignment

It is proposed that the WHO 6th IS FVIII/VWF Plasma (07/316) be assigned a value for VWF propeptide of 1.03 IU per ampoule.

Responses from study participants and the SSC/ISTH von Willebrand factor sub-committee

Responses in agreement with the proposal for value assignment of the WHO 6th IS FVIII/VWF Plasma have been obtained from all 13 collaborative study participants. In addition the report has been circulated to 8 co-chairs and 16 experts associated with the SSC/ISTH von Willebrand factor sub-committee and all have agreed to the proposal. It was subsequently endorsed at the ISTH/SSC Sub-committee Meeting, held in Kyoto, Japan on 23-24 July 2011.

Stability Studies

Accelerated degradation study for VWFpp in the WHO 6th IS FVIII/VWF Plasma (07/316)

Stability of VWFpp in the WHO 6th IS FVIII/VWF Plasma (07/316) has been assessed in an accelerated degradation study which allows the estimation of predicted loss per year based on the observed loss occurring in ampoules stored at elevated temperatures (9). One laboratory (number 5) has measured the residual potencies of ampoules stored at +4, +20 and +37 ^{o}C for 2 years 9 months relative to ampoules stored at -20 ^{o}C (Table 6). The results indicate that

VWFpp, in the WHO 6th IS, is extremely stable when stored at -20 °C with a mean loss of 0.04 % per year.

Stability of VWFpp in the WHO 6th IS FVIII/VWF Plasma (07/316) after reconstitution.

The stability of VWFpp in the reconstituted standard has been investigated in order to provide a guide to users. Results from two independent tests have indicated a mean of 103% of the zero time VWFpp concentration remains after storage of the reconstituted standard in a plastic tube for 4 hours on melting ice. The stability of VWFpp after reconstitution is therefore consistent with the details already provided in the Instructions for Use.

References

1 Haberichter SL, Balistreri M, Christopherson P, Morateck P, Gavazova S, Bellissimo DB, Manco-Johnson MJ, Cox Gill J, Montgomery RR (2006) Assay of the von Willebrand factor (VWF) propeptide to identify patients with type 1 von Willebrand disease with decreased VWF survival. Blood 108; 3344-51

2 van Genderen PJ, Boertjes RC, van Mourik JA (1998) Quantitative analysis of von Willebrand factor and its propeptide in plasma in acquired von Willebrand syndrome. Thromb Haemost 80; 495-8

3 Hubbard AR (2006) von Willebrand Factor standards for plasma and concentrate testing. Semin Thromb Hemost 32; 522-8

4 Recommendations for the preparation, characterization and establishment of international and other biological reference standards (revised 2004). WHO Technical Report Series 2007. Geneva, Switzerland: WHO 2007; 932, 73-131 http://www.who.int/bloodproducts/publications/TRS932Annex2_Inter_biolefstandards rev2004.pdf

5 Hubbard AR, Hamill M, Beeharry M, Bevan S, Heath AB (2009) Value Assignment of the Proposed 6th International Standard for Blood Coagulation Factor VIII and von Willebrand Factor in Plasma, Human (07/316). WHO/BS/09.2116 http://whqlibdoc.who.int/hq/2009/WHO_BS_09.2116_eng.pdf

6 Finney DJ (1978) Statistical methods in biological assay. 3rd edition Charles Griffin. London.

7 Grubbs F (1969) Procedures for detecting outlying observations in samples. Technometrics 11; 1-21

8 Kirkwood TBL (1979) Geometric means and measures of dispersion. Biometrics 35; 908-9

9 Kirkwood TBL & Tydeman MS (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

Acknowledgements

This collaborative study was organised with the assistance of the Working Party on VWFpp Standardization of the SSC Sub-committee on von Willebrand factor. The authors of the report and the members of the Working Party acknowledge the efforts of the participating laboratories and the support of the chair and members of the SSC Sub-committee on von Willebrand factor.

Instructions for Use

The revised Instructions for Use are found in Appendix 4.

TABLE 1 Estimates for VWFpp (units/ml) in WHO 6th IS Plasma and SSC Lot#3 relative to local reference preparations

Lab No.	WHO IS Plasma vs Local Reference		SSC Lot #3 vs Local Reference	
	Mean	GCV%	Mean	GCV%
1 (ng/ml)	510.82	4.1	493.50	1.8
2	1.02	2.6	1.02	3.4
3	1.21	5.5	1.21	2.8
4	0.92	2.2	0.92	7.1
5	1.05	2.4	0.98	3.9
6	0.97	3.4	0.95	4.3
7	0.94*	4.6*	0.94*	2.7*
8	1.05	7.7	1.05	7.6
9	1.13	4.3	1.14	5.6
10 (nM)	5.59	9.5	5.80	5.2
11	1.03	1.8	0.97	17.0
12	1.06*	8.1*	1.04*	12.3*
13	1.01*	5.9*	0.97*	3.5*
Overall mean GCV%	1.03 (n=11) 8.1		1.01 (n=11) 8.8	

Overall mean values exclude results from Labs 1 & 10 which are not in units/ml;
*** - mean of values calculated by participants relative to the GTI kit controls**

TABLE 2 Estimates for VWFpp (units/ml) in SSC Lot #3 relative to the WHO 6th IS Plasma (arbitrary 1.0 unit/ml)

Lab No.	SSC Lot #3 vs WHO IS Plasma	
	Mean	GCV%
1	0.96	4.1
2	1.00	3.9
3	1.00	7.2
4	1.00	7.8
5	0.94	2.7
6	0.99	7.2
7	1.00	6.7
8	1.00	1.0
9	1.01	2.9
10	1.03	12.7
11	0.94	16.7
12	0.96	11.4
13	0.96	4.9
Overall mean GCV%	0.98 (n=13) 3.0	

NOTE: All labs included in overall mean in Table 2

TABLE 3 Estimates for VWF:Ag (IU/ml) in WHO 6th IS Plasma and SSC Lot#3 relative to local reference preparations

Lab No.	WHO IS Plasma vs Local Reference		SSC Lot #3 vs Local Reference	
	Mean	GCV%	Mean	GCV%
1	0.99	2.8	1.10	4.3
2	0.92	2.1	1.03	3.6
3	1.00	3.3	1.16	1.9
4	1.09	6.1	1.21	1.8
5	0.78	3.3	0.87	5.0
6	1.04	16.7	1.22	10.0
7	0.73*	5.3*	0.85*	4.1*
8	0.87	4.4	1.06	4.7
9	0.96	0.6	1.01	1.5
10 (ug/ml)	11.13	6.0	12.92	2.6
11	0.96	6.3	1.05	10.7
12	0.96*	23.9*	1.09*	5.4*
13	0.89*	3.3*	0.99*	3.0*
Overall mean GCV%	0.93 (n=12) 12.2		1.05 (n=12) 12.0	

Overall mean values exclude results from Lab 10 which are not in IU/ml;
*** - mean of values calculated by participants relative to the GTI kit controls**

TABLE 4 Estimates for VWF:Ag (IU/ml) in SSC Lot #3 relative to WHO 6th IS Plasma (assigned 1.00 IU/ml)

Lab No.	SSC Lot #3 v WHO IS Plasma	
	Mean	GCV%
1	1.11	1.8
2	1.12	4.5
3	1.16	2.1
4	1.11	5.6
5	1.11	5.2
6	1.16	12.2
7	1.16	8.2
8	1.22	6.9
9	1.06	1.7
10	1.16	4.5
11	1.10	5.4
12	1.14	5.5
13	1.12	4.5
Overall mean GCV%	1.13 (n=13) 3.5	

NOTE: All labs included in overall mean in Table 4

TABLE 5 Ratios of VWFpp/VWF:Ag for WHO 6th IS Plasma and SSC Lot #3

Lab No.	WHO IS Plasma vs Local Reference	SSC Lot #3 vs Local Reference	SSC Lot #3 vs WHO IS Plasma
1	-	-	0.865
2	1.109	0.990	0.893
3	1.210	1.040	0.862
4	0.844	0.760	0.901
5	1.346	1.126	0.847
6	0.933	0.779	0.853
7	1.288	1.106	0.862
8	1.207	0.991	0.820
9	1.177	1.129	0.953
10	-	-	0.888
11	1.073	0.924	0.855
12	1.104	0.954	0.842
13	1.135	0.980	0.857
Overall mean GCV%	1.120 (n=11) 14.5	0.972 (n=11) 14.3	0.868 (n=13) 3.8

Ratios relative to the local reference materials were calculated using the mean laboratory values from Tables 1 and 3. Ratios for SSC Lot #3 relative to the WHO IS Plasma were calculated using the mean laboratory values from Tables 2 and 4.

TABLE 6 Accelerated degradation study for VWFpp in the WHO 6th IS FVIII/VWF Plasma (07/316).

Storage temperature (°C)	Mean residual potency after 2 years 9 months storage* (as % of -20 °C ampoules)	Mean predicted loss (% per year)
-20	------	0.04 %
+4	98.9 %	0.62 %
+20	92.3 %	2.87 %
+37	70.7 %	11.82%

Mean residual potency for ampoules stored at elevated temperatures for 2 years 9 months and mean predicted loss (% per year). *Results are means of 4 independent estimates

Figure 1 Mean laboratory estimates for VWFpp in the WHO 6th IS Plasma relative to the local reference materials expressed as percentage of the overall mean value

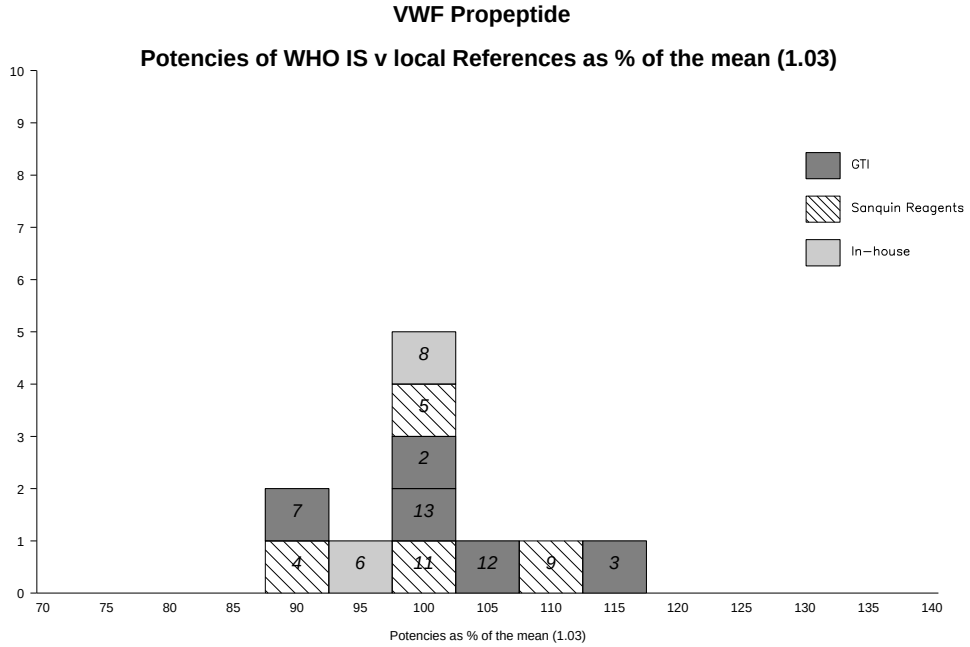


Figure 2 Mean laboratory estimates for VWFpp in the SSC Lot #3 relative to the local reference materials expressed as percentage of the overall mean value

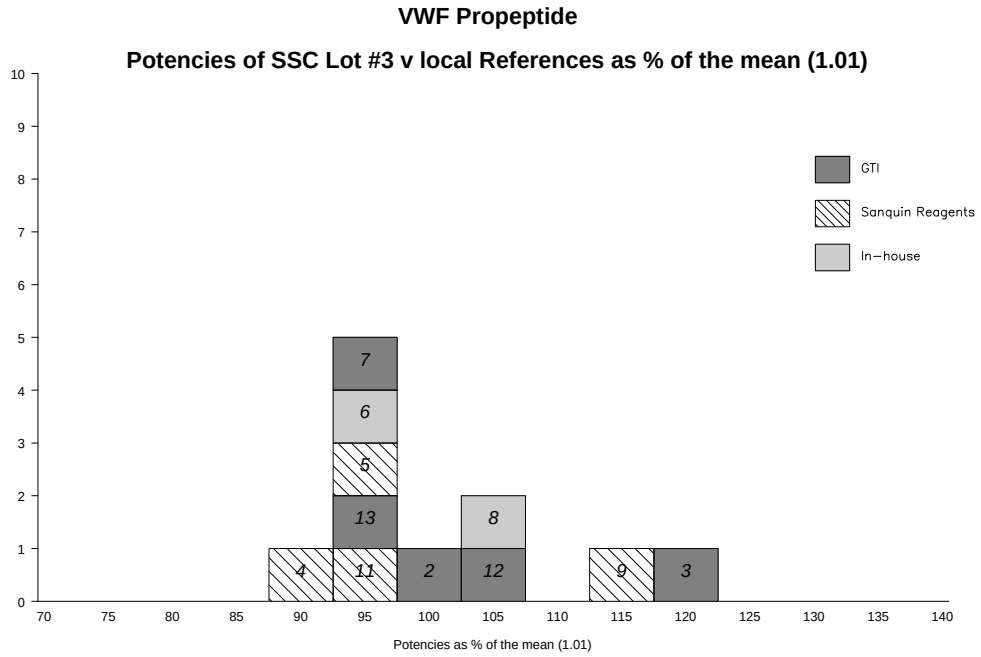
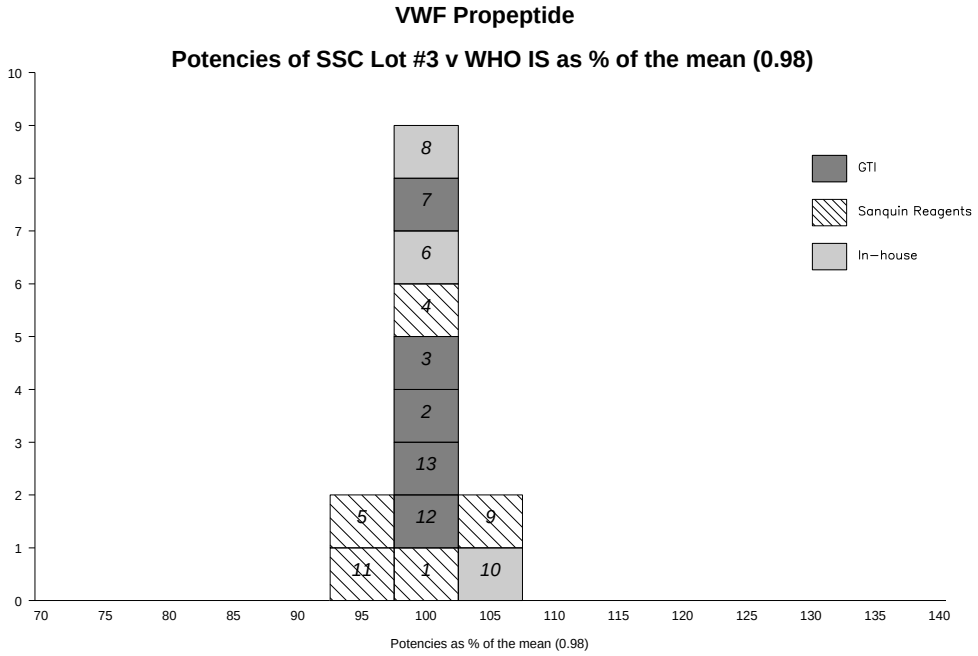


Figure 3 Mean laboratory estimates for VWFpp in the SSC Lot #3 relative to the WHO 6th IS Plasma expressed as percentage of the overall mean value



Appendix 1 Details of Methods and Local Reference Materials

Lab No	VWF Propeptide		VWF:Antigen	
	Method	**Local Reference**	**Method**	**Local Reference**
1	Sanquin Mouse Mabs	recomb VWFpp 3.525 mg/ml	Dako Rabbit polyclonal	WHO 1st IS Conc 11.0 IU/ml
2	GTI kit Mabs	single kit calibrator 0.93 U/ml	GTI kit	single kit calibrator 0.97 IU/ml
3	GTI kit Mabs	frozen normal pool 1.03 U/ml	GTI kit	frozen normal pool 1.03 IU/ml
4	Sanquin Mouse Mabs	frozen normal pool 1 U/ml	Dako Rabbit polyclonal	frozen normal pool 1.51 IU/ml
5	Sanquin Mouse Mabs	frozen normal pool 1.00 U/ml	Mouse Mab Rabbit polyclonal	frozen normal pool 0.98 IU/ml
6	In house Mouse Mab Rabbit polyclonal	frozen normal pool 1 U/ml	In house Rabbit polyclonal	WHO 1st IS Conc 11.0 IU/ml
7*	GTI kit Mabs	*single kit calibrator	GTI kit	*single kit calibrator
8	In house	SSC #3 (frozen) 1.06 U/ml	In house	SSC #3 (frozen) 1.06 IU/ml
9	Sanquin Mouse Mabs	lyoph normal pool 1.00 U/ml	Siemens turbidimetric	lyoph normal pool 0.92 IU/ml
10	Rabbit polyclonal	frozen normal pool 6.3 nM	Dako Rabbit polyclonal	frozen normal pool 13 ug/ml
11	Sanquin Mouse Mabs	frozen normal pool 1.00 U/ml	Dako Rabbit polyclonal	frozen normal pool 1.02 IU/ml
12*	GTI kit Mabs	*frozen normal pool unassigned	GTI kit	*frozen normal pool unassigned
13*	GTI kit Mabs	*frozen normal pool unassigned	GTI kit	*frozen normal pool unassigned

Mabs - monoclonal antibodies; * - results estimated by participants relative to kit controls rather than local reference materials were included in the study analysis

Appendix 2 List of Participants

Dr C Caron, Laboratoire d'Hématologie, Centre de Biologie Pathologie, CHR, Lille, France

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Dr D Lillicrap, Dept of Pathology & Molecular Medicine, Queen's University, Kingston ON, Canada

Dr Michelle Stapleton, GTI Diagnostics, Waukesha WI, USA

Dr B Samor, LFB Biotechnologies, Lille, France

Dr B Konkle, Hemostasis Reference Laboratory, Puget Sound Blood Center, Seattle WA, USA

Dr K Mertens/Ms J M Rentenaar, Sanquin Blood Supply Foundation, Amsterdam, The Netherlands

Ms A Riddell, Haemophilia Centre & Thrombosis Unit, Royal Free Hospital, London, UK

Prof P Sie, Lab Hématologie, Hôpital Rangeuil, Toulouse, France

Dr J Patzke, Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany

Dr RR Montgomery, Blood Research Institute, Milwaukee WI, USA

Prof Dr PG de Groot/Dr M Roest, UMC Utrecht, The Netherlands

Appendix 3 Study Protocol

STANDARDIZATION OF VWF PROPEPTIDE ESTIMATION

OBJECTIVES

- evaluation of inter-laboratory variability of VWFpp and VWF:Ag estimates and VWFpp/VWF:Ag ratio in the WHO 6th IS FVIII/VWF Plasma and the SSC/ISTH Secondary Coagulation Standard Lot #3 (SSC Lot #3), relative to local in house reference preparations
- assignment of value for VWFpp to WHO 6th IS FVIII/VWF Plasma and SSC Lot #3 by consensus mean of values relative to local in house reference preparations (subject to acceptable inter-laboratory agreement)

1 SAMPLES INCLUDED IN THE ASSAYS

- WHO 6th IS FVIII/VWF Plasma (07/316) – 10 ampoules provided
- SSC/ISTH Secondary Coagulation Standard Lot #3 - 10 vials provided
- Your local in house reference for VWFpp - 4 aliquots *(for VWFpp assays only)*
- Your local in house reference for VWF:Ag - 4 aliquots *(for VWF:Ag assays only)*

2 STORAGE AND RECONSTITUTION OF WHO 6th IS and SSC Lot #3

Store the unopened ampoules/vials of WHO 6th IS and SSC Lot #3 at -20°C or below. Allow the ampoules/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/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 contents to stoppered plastic tubes, store at 4°C and begin the assays as soon as possible.

3 STUDY PLAN

You are requested to carry out a total of 4 assays for VWFpp and 4 assays for VWF:Ag using fresh ampoules/vials of WHO 6th IS and SSC Lot #3 and fresh samples of your local in house references in each assay. In order to address the day-to-day variability please carry out the assays over at least 2 different days. At least 4 dilutions of each preparation should be tested, in replicate, within each assay to allow the generation of a dose-response relationship for each preparation. Dilutions should be chosen to fall on the steepest (most sensitive) portion of the dose-response curve and to provide maximum overlap between the different preparations. Please add the samples to the microplate in a balanced fashion which should be varied between the different assays, eg

LocalRef WHO6thIS SSCLot#3 SSCLot#3* WHO6thIS* LocalRef*

where each name refers to a set of 4 different dilutions (eg. 1/100, 1/200, 1/400, 1/800) and **WHO6thIS** and **WHO6thIS*** etc. refer to independent sets of dilutions (replicates)

4 RESULTS

Please insert the raw data and the calculated estimates from your assays on the EXCEL results sheets (sent by e-mail) to allow centralised analysis. Please return the results sheets and questionnaire on methodology to: Dr A Hubbard, Haemostasis Section, Biotherapeutics Group, NIBSC, Blanche Lane, South Mimms, Potters Bar, Herts. EN6 3QG United Kingdom Fax: +44 1707 641050 E-mail: anthony.hubbard@nibsc.hpa.org.uk

Appendix 4 Instructions for Use

WHO International Standard
6th INTERNATIONAL STANDARD FACTOR VIII AND VON WILLEBRAND FACTOR IN PLASMA
NIBSC code: 07/316
Instructions for use
(Version 1.00, Dated 04/11/2009)

1. INTENDED USE

The WHO 6th International Standard for Factor VIII and von Willebrand Factor in plasma was established by the Expert Committee on Biological Standardisation (ECBS) of the World Health Organisation (WHO) in October 2009 and details of the preparation and value assignment are available in document WHO/BS/09.2116. Assignment of a value for an additional analyte, von Willebrand factor propeptide, was agreed by WHO ECBS in October 2011 as described in document WHO/BS/11.****. The preparation consists of glass ampoules (coded 07/316) containing 1 ml aliquots of pooled normal human plasma, freeze-dried. The International Standard (IS) has values assigned for the following analytes:

Factor VIII Clotting activity	- FVIII:C
Factor VIII Antigen	- FVIII:Ag
von Willebrand Factor Antigen	- VWF:Ag
von Willebrand Factor Ristocetin Cofactor function	- VWF:RCo
von Willebrand Factor Collagen Binding function	- VWF:CB
von Willebrand Factor Propeptide	- VWFpp

The standard is intended to be used for the estimation of these analytes in human plasma. For the estimation of FVIII:C in therapeutic concentrates it is recommended that the current WHO International Standard Factor VIII Concentrate is used. For the estimation of VWF:Ag and VWF:RCo in therapeutic concentrates it is recommended that the current WHO International Standard von Willebrand Factor Concentrate is used. The WHO 6th International Standard Factor VIII and von Willebrand Factor in plasma (07/316) should not be used for the estimation of VWF:CB in therapeutic concentrates.

2. CAUTION

<u>This preparation is not for administration to humans</u>.

The preparation contains material of human origin, and either the final product or the source materials, from which it is derived, have been tested and found negative for HBsAg, anti-HIV and HCV RNA. As with all materials of biological origin, this preparation should be regarded as potentially hazardous to health. It should be used and discarded according to your own laboratory's safety procedures. Such safety procedures should include the wearing of protective gloves and avoiding the generation of aerosols. Care should be exercised in opening ampoules or vials, to avoid cuts.

3. UNITAGE

The following assigned values (except for VWFpp) were determined by comparison relative to the WHO 5th International Standard Factor VIII and von Willebrand Factor in plasma (02/150) in an international collaborative study involving 44 laboratories in 14 countries. The value for VWFpp was assigned relative to local reference materials in a collaborative study involving 13 laboratories. The overall mean values assigned to each ampoule of the WHO 6th IS are as follows:

FVIII:C	0.68 IU per ampoule
FVIII:Ag	1.04 IU per ampoule
VWF:Ag	1.00 IU per ampoule
VWF:RCo	0.87 IU per ampoule
VWF:CB	1.03 IU per ampoule
VWFpp	1.03 IU per ampoule

Uncertainty: the assigned unitage does not carry an uncertainty associated with its calibration. The uncertainty may therefore be considered to be the variance of the ampoule content and was determined to be +/- 0.118%.

4. CONTENTS

Country of origin of biological material: United Kingdom.
The WHO 6th International Standard was prepared at the National Institute for Biological Standards and Control in March 2008 from a pool of 23 litres of plasma collected from 80 donors. Blood was collected into CPD-A anticoagulant (63 ml CPD-A +420 ml blood) and each unit underwent leuco-depletion by filtration. The individual donations underwent two centrifugation cycles before being stored frozen at -70 °C until the day of filling. Plasma units were thawed on the day of filling by immersion in waterbaths at 37 °C. The pooled plasma was buffered by the addition of HEPES to a final concentration of 0.04 mol/l. The pooled plasma was kept at 4 °C throughout distribution into approximately 20,000 glass ampoules and then freeze-dried under conditions used for international biological standards (1). The mean liquid filling weight was 1.1056 g (range 1.1010 g to 1.1095 g) and the coefficient of variation was 0.118% based on 786 check-weight ampoules. Estimates of residual moisture after freeze-drying gave a mean value of 0.30% (n=12). Estimates of oxygen in the headspace gave a mean value of 0.13% (n=12).

5. STORAGE

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

6. DIRECTIONS FOR OPENING

DIN ampoules have an 'easy-open' coloured stress point, where the narrow ampoule stem joins the wider ampoule body.
Tap the ampoule gently to collect the material at the bottom (labeled) end. Ensure that the disposable ampoule safety breaker provided is pushed down on the stem of the ampoule and against the shoulder of the ampoule body. Hold the body of the ampoule in one hand and the disposable ampoule breaker covering the ampoule stem between the thumb and first finger of the other hand. Apply a bending force to open the ampoule at the coloured stress point, primarily using the hand holding the plastic collar.
Care should be taken to avoid cuts and projectile glass fragments that might enter the eyes, for example, by the use of suitable gloves and an eye shield. Take care that no material is lost from the ampoule and no glass falls into the ampoule. Within the ampoule is dry nitrogen gas at slightly less than atmospheric pressure. A new disposable ampoule breaker is provided with each DIN ampoule.

7. USE OF MATERIAL

<u>No attempt should be made to weigh out any portion of the freeze-dried material prior to reconstitution.</u>
Dissolve the total contents of the ampoule by adding 1.0 ml of distilled water, using gentle shaking, then transfer the contents to a plastic tube. Although studies have shown the reconstituted standard to be stable for up to 3 hours when kept on melting ice it is recommended that assays of FVIII:C and VWF:RCo be carried out as soon as possible after reconstitution. The use of frozen aliquots for FVIII:Ag, VWF:Ag and VWFpp estimation should be validated locally. It is not recommended that frozen aliquots are used for FVIII:C, VWF:RCo or VWF:CB estimation.

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.

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NIBSC
A centre of the Health Protection Agency

Health Protection Agency

9. REFERENCES

1. Campbell PJ. Procedures used for the production of biological standards and reference preparations. Journal of Biological Standardization (1974) 2, 259-267.

10. ACKNOWLEDGEMENTS

Are made to the participants in the collaborative study, to the staff of the Standards Processing Division (NIBSC) and to the chairs and members of the SSC/ISTH sub-committees for FVIII/FIX and von Willebrand factor for their support.

11. FURTHER INFORMATION

Further information can be obtained as follows;
This material:
enquiries@nibsc.hpa.org.uk
WHO Biological Standards:
Http://www.who.int/biologicals/en/
JCTLM Higher order reference materials:
Http://www.bipm.org/en/committees/jc/jctlm/
Derivation of International Units:
http://www.who.int/biologicals/reference_preparations/en/
Ordering standards from NIBSC:
Http://www.nibsc.ac.uk/products/ordering_information/frequently_asked_questions.aspx
NIBSC Terms & Conditions:
Http://www.nibsc.ac.uk/terms_and_conditions.aspx

12. CUSTOMER FEEDBACK

Customers are encouraged to provide feedback on the suitability or use of the material provided or other aspects of our service. Please send any comments to enquiries@nibsc.hpa.org.uk

13. CITATION

In all publications, including data sheets, in which this material is referenced, it is important that the preparation's title, its status, the NIBSC code number, and the name and address of NIBSC are cited and cited correctly.

14. MATERIAL SAFETY SHEET

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

Toxicological properties	
Effects of inhalation:	Not established, avoid inhalation
Effects of ingestion:	Not established, avoid ingestion
Effects of skin absorption:	Not established, avoid contact with skin

Suggested First Aid	
Inhalation:	Seek medical advice
Ingestion:	Seek medical advice
Contact with eyes:	Wash with copious amounts of water. Seek medical advice
Contact with skin:	Wash thoroughly with water.

Action on Spillage and Method of Disposal
Spillage of contents should be taken up with absorbent material wetted with an appropriate disinfectant. Rinse area with an appropriate disinfectant followed by water. Absorbent materials used to treat spillage should be treated as biological waste.

15. LIABILITY AND LOSS

Information provided by the Institute is given after the exercise of all reasonable care and skill in its compilation, preparation and issue, but it is provided without liability to the Recipient in its application and use.
It is the responsibility of the Recipient to determine the appropriateness of the standards or reference materials supplied by the Institute to the Recipient ("the Goods") for the proposed application and ensure that it has the necessary technical skills to determine that they are appropriate. Results obtained from the Goods are likely to be dependant on conditions of use by the Recipient and the variability of materials beyond the control of the Institute.
All warranties are excluded to the fullest extent permitted by law, including without limitation that the Goods are free from infectious agents or that the supply of Goods will not infringe any rights of any third party.
The Institute shall not be liable to the Recipient for any economic loss whether direct or indirect, which arise in connection with this agreement.
The total liability of the Institute in connection with this agreement, whether for negligence or breach of contract or otherwise, shall in no event exceed 120% of any price paid or payable by the Recipient for the supply of the Goods.
If any of the Goods supplied by the Institute should prove not to meet their specification when stored and used correctly (and provided that the Recipient has returned the Goods to the Institute together with written notification of such alleged defect within seven days of the time when the Recipient discovers or ought to have discovered the defect), the Institute shall either replace the Goods or, at its sole option, refund the handling charge provided that performance of either one of the above options shall constitute an entire discharge of the Institute's liability under this Condition.

16. INFORMATION FOR CUSTOMS USE ONLY

Country of origin for customs purposes*: United Kingdom * Defined as the country where the goods have been produced and/or sufficiently processed to be classed as originating from the country of supply, for example a change of state such as freeze-drying.
Net weight: 0.093 g
Toxicity Statement: Non-toxic
Veterinary certificate or other statement if applicable. **Attached:** No