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Value Assignment of the proposed WHO 1st International Standard ADAMTS13, Plasma (12/252)

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NOTE:
This document has been prepared for the purpose of inviting comments and suggestions on the proposals contained therein, which will then be considered by the Expert Committee on Biological Standardization (ECBS). Comments MUST be received by **4 October 2014** and should be addressed to the World Health Organization, 1211 Geneva 27, Switzerland, attention: Technologies, Standards and Norms (TSN). Comments may also be submitted electronically to the Responsible Officer: **Dr David Wood** at email: woodd@who.int.

Summary

A collaborative study involving 32 laboratories from 14 countries has been undertaken to assign values for ADAMTS13 function and antigen to the proposed WHO 1st International Standard (IS) ADAMTS13 plasma (coded 12/252). Value assignment was based on assays relative to local pooled normal plasma preparations arbitrarily assigned 1.0 unit per ml. Most laboratories used either a Fluorescence Resonance Energy Transfer (FRET) assay (n=18) or an activity ELISA (n=9) to measure ADAMTS13 function and all laboratories used ELISA for antigen measurement. The candidate WHO IS was included in the study as coded duplicates (samples A & B). Comparison of the candidate WHO IS (samples A & B) with the local normal pools was associated with a high degree of validity in terms of parallelism of the dose-response relationships with only 9/117 function assays and 8/58 antigen assays excluded because of non-parallelism. Estimates of ADAMTS13 function for samples A & B were not significantly different and there was also no significant difference between the results by FRET and the activity ELISA. Combination of all results for function gave an overall mean of 0.91 units/ml for the candidate WHO IS with low inter-laboratory variability (GCV) of 12.4%. For estimates of ADAMTS13 antigen in the coded duplicates of the candidate WHO IS (samples A & B) there was also no significant difference and combination of all results gave an overall mean of 0.92 units/ml with inter-laboratory variability (GCV) of 16.3%. Two samples (C & D) from a patient with acquired ADAMTS13 deficiency due to an inhibitory autoantibody were also included in the study. The level of ADAMTS13 in sample C was below the limit of detection for assays of function in many cases (21/32) and calculated estimates were only possible in 11 laboratories. However, 31/32 results were consistent with a severe deficiency below 0.1 units/ml. Patient sample D contained a higher level of ADAMTS13 than sample C and only 8/32 data sets from the function assays were not amenable to quantification. The overall mean estimate for function in sample D was 0.15 units/ml with 23/24 laboratories agreeing levels below 0.3 units/ml. Ratios of function to antigen for samples C and D were greatly reduced at 0.11 and 0.24 respectively compared to normal plasma (0.99). This finding together with the large inter-laboratory variability of estimates for the patient samples is most probably related to the presence of circulating antibody-ADAMTS13 complexes. The availability of a common reference material (proposed WHO IS) could help to identify the methodological issues responsible for this variability. Assays of recombinant ADAMTS13 (sample E) indicated valid comparison of dose-response relationships with normal plasma (proposed WHO IS) but large inter-laboratory of estimates for both function and antigen. This could indicate that the proposed WHO IS is not suitable for the assay of recombinant ADAMTS13.

Proposal

It is proposed that the preparation coded 12/252 is endorsed as the WHO 1st IS ADAMTS13, plasma with assignment of the following consensus mean values:

Function: 0.91 IU per ampoule
Antigen: 0.92 IU per ampoule

Responses and comments from study participants and SSC experts

Responses have been obtained from the 32 participants and all have approved the proposed assigned values for ADAMTS13 function and antigen. Two additional comments were received from the participants:
1) recommended a future study to evaluate the sensitivity of activity measurements.
Response: the development of the WHO 1st IS Plasma is the first step in the standardisation of ADAMTS13. Further studies will be necessary to investigate the sources of inter-laboratory variability and sensitivity issues.
2) recommended that the sources of reagents for the function measurements be added.
Response: this has been included as a footnote to Appendix 3.

In addition 13 SSC expert reviewers have also approved the proposed assigned values for function and antigen. The following comments were received:
3) the high GCVs for the patient samples are an issue of concern; I am confident that availability of an appropriate International Standard will help the issue further.
No response necessary.
4) recommended to change from ADAMTS13 "function" to "activity" in the report.
Response: this was not raised by any other reviewers and is a minority view. No change made to the report.
5) the International Standard may not be appropriate for the measurement of recombinant ADAMTS13.
Response: this is a valid comment in view of the discrepancy between methods and the large inter-laboratory variability.

The proposal to endorse the preparation coded 12/252 as the WHO 1st IS ADAMTS13 Plasma was approved at the SSC Board Meeting, held in Milwaukee WI, on 23 June 2014.

Introduction and objectives of the study

ADAMTS13 (**A** **D**isintegrin **A**nd **M**etalloprotease with **T**hrombo**S**pondin type 1 motifs **13**), also known as "von Willebrand factor (VWF) cleaving protease" is responsible for modulating the range of multimeric species of VWF in the circulation. Acquired or congenital deficiency of ADAMTS13 is associated with the circulation of ultra-large multimers of VWF which can lead to thrombotic thrombocytopoenic purpura (TTP) characterised by disseminated platelet aggregation, microvascular thrombosis, severe platelet deficiency, red cell hemolysis and organ damage. Measurement of ADAMTS13 in plasma is a crucial component in the diagnosis and treatment of TTP. Numerous methods, both commercial and "in house", are available for the estimation of ADAMTS13 function and antigen but there is currently no internationally accepted unitage to support harmonisation of measurement between laboratories. The primary objective of the current project is to develop the WHO 1st International Standard ADAMTS13, plasma with assigned values for ADAMTS13 function and antigen. Establishment of the WHO 1st IS will provide long-term continuity and stability for the ADAMTS13 unit and a traceable route for the calibration of all secondary working standards. Patient plasma samples and a preparation of recombinant ADAMTS13 were also included in the collaborative assays in order to assess testing relative to the proposed WHO 1st IS.

The unitage for ADAMTS13

Tests for ADAMTS13 are established in many laboratories and the majority of results for function are reported relative to local pooled normal plasmas which have arbitrarily assigned values either in units or percentage (where 1 unit per ml or 100% is equivalent to the concentration of ADAMTS13 in pooled normal plasma). Some measurements of ADAMTS13 antigen are reported in ng per ml relative to commercial reference preparations. However, value assignment of the WHO IS for antigen in ng per ml would be problematic since the consensus mean approach to value assignment is incompatible with an absolute SI mass unit. In common with other WHO IS for plasma proteins it is therefore proposed that an International Unit (IU) should be adopted for both ADAMTS13 activity and antigen where 1.0 IU per ml is equivalent to the concentration of ADAMTS13 in pooled normal plasma. The WHO IS Plasma will be assigned the consensus mean value of estimates calculated relative to the local pooled normal plasmas and, following the precedent for other standards in the haemostasis field, the assigned value will be labeled in IU.

Samples included in the collaborative study

Sample A: Proposed WHO 1st IS ADAMTS13, plasma (12/252).
The proposed WHO 1st IS was prepared from a pool of 38 donations from normal healthy donors (UK Blood Service) collected by conventional venepuncture into citrate-phosphate-dextrose-adenine anticoagulant. Each donation was checked and found negative for HBsAg, anti-HIV-1 and -2, anti-HCV, HCV RNA (mini-pool testing) and syphilis. Each donation underwent leuco-filtration before being centrifuged twice to remove all cellular components. The units were then frozen and stored at -70°C. Plasma was thawed on the day of filling, pooled and then buffered by the addition of HEPES (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]) to a final concentration of 40 mmol/l. One ml of the pooled plasma was dispensed into each of approximately 10,200 ampoules. Freeze-drying was performed in

accordance with the conditions required for International Standards (Campbell 1974). Details of the fill characteristics are given in the table below.

DETAILS OF CANDIDATE (12/252)	
Presentation	**sealed glass DIN ampoules**
Excipients/additives	**Recovered normal human plasma with additive HEPES (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]) to final concentration of 0.04 mol/l**
Liquid filling weight (g)	**Mean 1.0079 g**
Coefficient of variation of the liquid fill (%)	**0.245 % based on 420 check-weight ampoules**
Residual moisture after lyophilisation (%)	**Mean 0.35%, CV 18.8% (n = 12)**
Dry weight (mg)	**Mean 78.6 mg, CV 0.77% (n = 5)**
Headspace oxygen (%)	**Mean 0.29%, CV 31.7% (n = 12)**
Reconstitution volume and fluid	**1.0 ml distilled water**
Number of ampoules in stock	**10,000**
Manufacturing site	**NIBSC, Potters Bar, UK**
Custodian	**NIBSC, Potters Bar, UK**
Storage temperature	**-20 °C**

Sample B: Duplicate ampoules of the proposed WHO 1st IS ADAMTS13, plasma (12/252)

Sample C: Plasma sample from the spent plasma bag of plasma exchange session 1 from a patient with acquired ADAMTS13 deficiency due to an inhibitory autoantibody. This sample was lyophilised under the same conditions used for the candidate WHO IS (12/252). It was also tested and found negative for HAV RNA (by NAT), HBsAg, HCV RNA and antibodies to HIV-1 and -2.

Sample D: Plasma sample from the spent plasma bag of plasma exchange session 3 from a patient with acquired ADAMTS13 deficiency due to an inhibitory autoantibody. This sample was lyophilised under the same conditions used for the candidate WHO IS (12/252). It was also tested and found negative for HAV RNA (by NAT), HBsAg, HCV RNA and antibodies to HIV-1 and -2.

Sample E: Recombinant ADAMTS13.

Sample L: Local pooled normal plasma supplied by each participating laboratory. Details of local plasma pools are given in Appendix 3.

Participants and Study Design

Samples were despatched in September 2013 to 35 laboratories and results were received from 32 laboratories (14 countries) comprising 24 clinical laboratories, 6 manufacturers and 2 regulators (Appendix 1). Each laboratory was assigned a confidential code number which does not relate to the list order in Appendix 1. Participants were requested to follow their routine methodology as far as possible within the specified study protocol (Appendix 2). Each laboratory was requested to perform 4 independent assays for each method using fresh ampoules of Samples A to E and fresh samples of local pooled normal plasma (Sample L) in each assay. It was requested that the 4 assays be performed over at least 2 different days / sessions 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 and local pooled normal plasmas used by the participants are listed in Appendix 3. The methods used to measure ADAMTS13 function are summarised below. It is clear that most laboratories now perform assays utilising synthetic VWF A2 domain peptides as substrate (Fluorescence Resonance Energy Transfer (FRET) and activity ELISA). All estimates of antigen were obtained using ELISA techniques.

Method	**No. of datasets**
VWF A2 domain peptide substrate:	
FRET	19
Activity ELISA	10
Gold particle	1
Chemiluminescence	1
Full-length VWF substrate:	
Collagen Binding	2
Residual Ristocetin Cofactor / VWF activity	2

Statistical Analysis

All assays were analysed as multiple parallel line bioassays comparing response to log concentration (Finney, 1978). 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 assay the estimates for the proposed WHO IS Plasma (samples A and B) were calculated relative to the local pooled normal plasmas (sample L) using an arbitrary value of 1.0 unit/ml or the assigned value where available. The estimates for samples C, D, E were calculated relative to both the local pooled normal plasmas (sample L) and the proposed WHO IS Plasma (sample A) using the proposed assigned values for function and antigen. 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.

Where laboratories used more than one method, for function or antigen, the results from each method were treated as from different laboratories and identified with a letter suffix to the laboratory number. Differences in potency estimates between laboratories (outlier detection) were assessed using Grubb's test on the $\log_{10}$ potency estimates (Grubbs, 1969). Significant differences between data groups were assessed by paired "t" test or 2-sample "t" test on the $\log_{10}$ potency estimates. Intra- and inter-laboratory variability is expressed as the geometric coefficient of variation (GCV%) (Kirkwood, 1979).

Results were returned from 32 laboratories and comprised 35 data sets for ADAMTS13 function and 18 data sets for antigen.

ADAMTS13 function: Centralised parallel line analysis was not possible for the data returned by laboratories 8, 9, 15, 18 and 25 because of the use of unsuitable sample dilutions causing shallow dose-response relationships, responses exceeding the minimum or maximum ranges of detection or the failure to include sample L in the assay. For laboratories 8, 15 and 18 it was decided that estimates calculated by the participants could be normalised relative to sample L for inclusion in the tables of the results. It was not possible to analyse the single assays returned by laboratories 9 and 25 for inclusion in the study. Data from the remaining laboratories underwent parallel line analysis which included evaluation of the ratio of dose-response slopes as a criterion for validity. For the analysis of samples A and B relative to L there were 9 out of 117 assays with slope ratios outside the 80 - 125% range (single assays from laboratories 11, 12C, 17, 19, 21, 22, 31; 2 assays from lab 26); these assays have been excluded from the analysis.
Additional samples: Estimates for sample C could not be calculated for several laboratories because the responses were below the dose-response range for samples L and A. Of the remaining assays many (21 out of 35) were based on a single dilution and parallel line analysis was not possible. However, 14 assays used sufficient dilutions to allow the test for parallelism and 8 had slope-ratios outside the range of 80 - 125% that of sample A. The slopes of C were generally lower than for sample A with a geometric mean ratio across all assays of 81%.
For sample D 12 out of 91 assays were based on a single dilution and tests for parallelism were not possible. Tests for parallelism were possible for 79 assays and 29 had slope-ratios outside the range of 80 - 125% of sample A. The slopes of D were generally lower than sample A with a geometric mean ratio across all assays of 93%.
For sample E all 99 assays underwent tests for parallelism and 34 assays had slope ratios outside the range 80 - 125% of sample A. The geometric mean slope ratio across all assays was 107%. The large number of assays that appear non-parallel was mainly the result of inappropriate dilutions. Combined estimates for samples C, D and E were calculated using all of the assays relative to either samples A or L. Combined estimates relative to sample A were also calculated with the exclusion of assays where the slope ratios were outside the 80 - 125% range.

ADAMTS13 antigen: Centralised parallel line analysis was not possible for the data returned by laboratories 8, 9 and 25 because of the use of unsuitable sample dilutions or responses exceeding the minimum or maximum ranges of detection. For the single assay returned by laboratory 8 it was decided that estimates calculated by the participant could be normalised, relative to sample L, for inclusion in the tables of the results. It was not possible to analyse the single assays returned by laboratories 9 and 25 for inclusion in the study. Data from the remaining laboratories underwent parallel line analysis which included evaluation of the ratio of dose-response slopes as a criterion for validity. For the analysis of samples A and

B relative to L there were 8 out of 58 assays with slope ratios outside the 80 - 125% range (three assays from lab 13, two from lab 23 and one each from labs 1, 21 and 31); these assays have been excluded from the analysis.
Additional samples: For sample C there were 33 assays with enough data to calculate slopes and 11 assays had slopes outside the range of 80 - 125% with the slopes for C generally lower (flatter) than for A. The geometric mean slope ratio for all assays of C was 83%. For sample D there were 51 assays with enough data to calculate slopes and 13 assays had slopes outside the range of 80 - 125% with the slopes for D generally lower (flatter) than for A. The geometric mean slope ratio for all assays of D was 86%. For sample E there were 53 assays with enough data to calculate slopes and 22 assays had slopes outside the range of 80 - 125% primarily caused by the use of unsuitable dilution ranges. The geometric mean slope ratio for all assays of E was 87%. Combined estimates for samples C, D and E were calculated using all of the assays relative to either samples A or L. Combined estimates, relative to sample A, were also calculated with the exclusion of assays where the slope ratios were outside the 80 - 125% range.

Results

ADAMTS13 Function

Intra- and inter-laboratory variability of estimates.
The intra-laboratory (inter-assay) variability of estimates for the proposed WHO IS (samples A and B) relative to sample L was low with 31 out of 66 comparisons having a GCV of less than 5% and 54 out of 66 having a GCV less than 10%. Considering that all laboratories used a different local plasma pool (sample L) the variability between laboratories was also low with a GCV of 12.4% for the combined mean potency of the proposed WHO IS Plasma (Table 1).
Intra-laboratory variability was greater for samples C, D, E and this may be related to a lower degree of overlap of the dose-responses with samples A and L. Results for sample C were the most variable with intra-laboratory variability (GCV) exceeding 20% in 5 out of 9 laboratories compared to sample E where 16 out of 29 laboratories had variability less than 10%. Inter-laboratory variability for samples C, D and E was much larger than samples A and B, with GCVs of 120%, 54% and 47% respectively, relative to sample L (Tables 2 & 3).

Proposed WHO 1st IS Plasma (Samples A & B) relative to the Local Pooled Normal Plasma (Sample L) (Table 1 and Figure 1)
Samples A and B were coded duplicates of the proposed WHO IS Plasma. There were no outlying results detected for samples A or B at the 5% significance level. Mean laboratory estimates ranged from 0.73 to 1.21 units/ml for sample A and from 0.72 to 1.19 units/ml for sample B with identical overall combined mean values from all methods of 0.91 units/ml for both A and B. There was no significant difference between the estimates for samples A and B (paired t test $p = 0.281$). There was also no significant difference between estimates by the FRET method (mean 0.92 units/ml; n=18) and the activity ELISA method (mean 0.86 units/ml; n=9) ($p=0.181$). Combination of all mean laboratory estimates from samples A and B gave an overall mean value of 0.91 units/ml with inter-laboratory variability (GCV) of 12.4%.

Patient Samples C and D relative to the Local Pooled Normal Plasma (Sample L) and the Proposed WHO 1st IS (Sample A) (Tables 2 & 3 and Figures 2 & 3)
Many estimates for sample C were below the level of quantification relative to samples L and A. However, estimates from 11 laboratories which could be calculated ranged from 0.008 to 0.137 with an overall mean of 0.028 units/ml, relative to sample L, and from 0.011 to 0.11 with an overall mean of 0.030 units/ml, relative to sample A. The inter-laboratory variability (GCV) with all estimates was 120% relative to sample L and 95% relative to sample A. Exclusion of assays when the slope-ratios relative to sample A were outside 80 - 125% made little difference to the overall mean (0.029 units/ml) and inter-laboratory variability (GCV 85%).
Estimates for sample D were calculated from the results of 24 laboratories and ranged from 0.06 to 0.44 units/ml with an overall mean of 0.15 units/ml, relative to sample L, and from 0.04 to 0.32 units/ml with an overall mean of 0.15 units/ml relative to sample A. The inter-laboratory variability (GCV) was 54% and 63% for all estimates relative to samples L and A respectively. There was a significant difference between estimates by FRET methods and the activity ELISA measured relative to sample L ($p=0.043$) but not when measured relative to sample A ($p=0.165$). Exclusion of assays where the slope-ratios relative to sample A were outside 80 - 125% made no difference to the overall mean (0.15 units/ml) or inter-laboratory variability (GCV 63%).

Recombinant ADAMTS13 (Sample E) relative to the Local Pooled Normal Plasma (Sample L) and the Proposed WHO 1st IS (Sample A) (Tables 2 & 3 and Figure 4)
Estimates for sample E were calculated from the results of 29 laboratories and ranged from 21.4 to 112.4 units/ml with an overall mean of 56.1 units/ml, relative to sample L, and from 25.5 to 93.4 units/ml with an overall mean of 55.0 units/ml, relative to sample A. The inter-laboratory variability (GCV) was 47% and 45% for all estimates relative to samples L and A respectively. There was a significant difference between estimates by the FRET method and Exclusion of assays where the slope-ratios, relative to sample A, were outside 80 - 125% made little difference to the overall mean (57.7 units/ml) or inter-laboratory variability (GCV 39%).

ADAMTS13 Antigen

Intra- and inter-laboratory variability of estimates.
The intra-laboratory (inter-assay) variability of estimates for samples A and B relative to sample L was generally low with 12 out of 32 comparisons having a GCV of less than 5% and 20 out of 32 having a GCV less than 10%. Considering that all laboratories used a different local plasma pool (sample L) the variability between laboratories was low with a GCV of 16.3% for the combined mean potency of the proposed WHO IS Plasma (Table 4).

Intra-laboratory variability for samples C and D was similar to that for samples A and B with approximately half of the laboratories with GCVs less than 10%. The intra-laboratory variability for sample E was larger for some laboratories with 5/15 exceeding GCVs of 20%. Inter-laboratory variability for samples C, D and E was larger than samples A and B, with GCVs of 35%, 46% and 77% respectively relative to sample L (Tables 5 & 6).
Proposed WHO 1st IS Plasma (Samples A & B) relative to the Local Pooled Normal Plasma (Sample L) (Table 4 and Figure 5)
There were no outlying results detected for samples A or B at the 5% significance level. Mean laboratory estimates ranged from 0.72 to 1.17 units/ml for sample A and from 0.64 to

1.13 units/ml for sample B with combined mean values for sample A and sample B of 0.93 and 0.92 units/ml respectively. There was no significant difference between the estimates for samples A and B (paired t test, p = 0.443). Combination of all mean laboratory estimates for samples A and B gave a mean value of 0.92 units/ml with inter-laboratory variability (GCV) of 16.3%.

Patient Samples C and D relative to the Local Pooled Normal Plasma (Sample L) and the Proposed WHO 1st IS (Sample A) (Tables 5 & 6 and Figures 2 & 3)
Analysis of all estimates for sample C produced mean laboratory values ranging from 0.12 to 0.42 units/ml with an overall mean of 0.25 units/ml (n=14) relative to sample L and from 0.15 to 0.47 units/ml with a combined mean of 0.26 units/ml (n=14) relative to sample A. The inter-laboratory variability (GCV) with all estimates was 35% relative to sample L and 31% relative to sample A. Exclusion of assays when the slope-ratios relative to sample A were outside 80 - 125% made no difference to the overall mean (0.26 units/ml) and inter-laboratory variability (GCV 30%).

Mean laboratory estimates for sample D ranged from 0.27 to 1.00 units/ml with an overall mean of 0.63 units/ml (n=15) relative to sample L and from 0.33 to 0.83 units/ml with a combined mean of 0.63 units/ml (n=15) relative to sample A. The inter-laboratory variability (GCV) for all estimates was 46% relative to sample L and 32% relative to sample A. Exclusion of assays when the slope-ratios relative to sample A were outside 80 - 125% made little difference to the overall mean (0.64 units/ml) and inter-laboratory variability (GCV 30%).

Recombinant ADAMTS13 (Sample E) relative to the Local Pooled Normal Plasma (Sample L) and the Proposed WHO 1st IS (Sample A) (Tables 5& 6 and Figure 4)
Mean laboratory estimates for sample E ranged from 63 to 454 units/ml with an overall mean of 221 units/ml (n=16) relative to sample L and from 60 to 470 units/ml with a combined mean of 224 units/ml (n=16) relative to sample A. The inter-laboratory variability (GCV) for all estimates was 77% relative to sample L and 72% relative to sample A. Exclusion of assays when the slope-ratios relative to sample A were outside 80 - 125% made little difference to the overall mean (222 units/ml) and inter-laboratory variability (GCV 83%).

Ratio of ADAMTS13 function to antigen

Overall mean estimates for function and antigen in the proposed WHO 1st IS (sample A/B), relative to sample L, agreed very closely (0.91 vs 0.92 units/ml) giving a function/antigen ratio of 0.99 as would be expected when comparing normal pooled plasma samples (Table 7). However, mean estimates of antigen for the two patient samples were much greater than the mean estimates for function producing function/antigen ratios of 0.12 and 0.24 for samples C and D respectively. A similar discrepancy was also found with the recombinant ADAMTS13 (sample E) with a function/antigen ratio of 0.25.

Stability of the proposed WHO 1st IS ADAMTS13 Plasma

Accelerated degradation study
Stability of the proposed WHO 1st IS has been assessed in an accelerated degradation study which allows the calculation of predicted loss per year based on the observed loss occurring in

ampoules stored at elevated temperatures (Kirkwood and Tydeman 1984). The study involved the measurement of ADAMTS13 function by the activity ELISA method and ADAMTS13 antigen by conventional ELISA in different laboratories. The residual potencies of ampoules stored at -20, 4, 20, 37 and 45 °C after storage for 10 months are given in Table 8 expressed as a % relative to ampoules stored at -70 °C. Very little relative loss was observed for ampoules stored at -20 to +20 °C for both function and antigen. Predicted degradation rates for the storage of ampoules at -20, 4 and 20 °C are given in Table 9. The predicted loss for ampoules stored at the bulk storage temperature (-20 °C) is less than 0.1% loss per year consistent with extreme stability for the proposed standard. Stability testing will continue at further time points during the lifetime of the standard.

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. In common with previous WHO Plasma Standards 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. Results from three separate tests for ADAMTS13 function (Activity ELISA) have indicated that a mean of 97.3% of the starting concentration was retained after 4 hours for the freshly reconstituted standard when stored in melting ice in plastic tubes. This period is sufficient for numerous assays to be performed. The use of frozen aliquots of the proposed WHO 1st IS is not recommended.

Conclusions

Value assignment of the proposed WHO 1st IS ADAMTS13 Plasma (Samples A & B)
Samples A and B were coded duplicates of the proposed WHO 1st IS ADAMTS13 plasma (code 12/252) and there was no significant difference between estimates of A and B for either function or antigen. Assays of function and antigen for sample A/B, relative to the local pooled normal plasma (sample L), were associated with good validity in terms of parallelism of the dose-response relationships with mean slope ratios outside the 80 - 125% range for only 9 out 117 function assays and 8 out of 58 antigen assays. This good comparison between samples A/B and the local pools (L) is to be expected considering that they are both normal plasmas and cover a similar dose-response range.
Estimates of function and antigen in sample A/B were associated with low variability between assays (intra-laboratory) and also between laboratories (inter-laboratory). The overall inter-laboratory variability of 12.4% and 16.3% for function and antigen, respectively, is encouraging considering that each laboratory used a different local plasma pool (L). Comparison of the two predominant methods for function, FRET and activity ELISA, did not indicate any significant difference. Overall these results support a robust value assignment for the candidate sample A/B for ADAMTS13 function with a mean value of 0.91 IU per ampoule and for antigen with a mean value of 0.92 IU per ampoule.

Estimates on patient samples C and D
Patient Samples C and D were obtained from plasma exchange spent bags (plasma exchange session 1 and 3, respectively) of a patient with acquired ADAMTS13 deficiency due to an inhibitory autoantibody. They were included to compare potency estimation using different methods (e.g. function vs antigen) and different reference preparations (e.g. local plasma

pools vs proposed WHO IS). Results from all laboratories for ADAMTS13 function in sample C were consistent with a severe deficiency (31/32 estimates below 10% or 0.1 unit/ml). Quantitative analysis was not possible for sample C in several laboratories because of the low ADAMTS13 content which produced responses below the dose-response for sample A/B and the local plasma pools (sample L); these results were recorded as below the level of quantification. Results from quantifiable assays indicated function less than 0.14 units/ml relative to the local normal pools (sample L) and less than 0.11 relative to the proposed WHO IS (sample A). Levels of ADAMTS13 function in sample D were higher than sample C and this allowed a better overlap of dose-responses relative to reference samples L and A so facilitating more quantitative analysis than sample C. Overall, the quantitative estimates from 23/24 laboratories indicated ADAMTS13 function in sample D below 30% normal or 0.3 units/ml.

The very large inter-laboratory variability for estimates of function in sample C (GCVs ~100%) is a common finding when results are around the limit of detection and should not be considered representative of true variability of function assays. However, it is clear from the results of sample D that there is significant inter-laboratory variability for the function methods (GCV 60%) even when measured relative to a common standard, the proposed WHO IS. This finding indicates that inter-laboratory variability is not primarily caused by the use of different local plasma references but may be due to methodological differences between laboratories. A similar conclusion has been made in other multi-centre studies on the measurement of ADAMTS13 function in patient/normal plasma mixtures using a range of methods including collagen binding, immunoblotting and FRET (Tripodi et al, 2004; 2008). The inter-laboratory variability for the patient samples (C and D), in the present study, was greater than that reported for the patient/normal plasma mixtures by Tripodi et al (2008) and this may be related to the presence of immune complexes in samples C and D. The availability of a common reference preparation, the WHO IS should facilitate future studies to identify the factors causing this variability which may be masked when different local plasma references are used.

The presence of immune complexes in the patient samples is also consistent with the ratios of function to antigen which were greatly reduced compared to the normal plasma in the proposed WHO IS. Similar discrepancies between function and antigen measurements have been previously reported on patients with acquired ADAMTS13 deficiency due to autoantibodies and it has been proposed that this may be caused by the presence of circulating ADAMTS13-autoantibody complexes where ADAMTS13 activity is inhibited but which may still be detected by assays for antigen (Rieger et al, 2006).

Estimates on recombinant ADAMTS13 (Sample E)
Sample E was a preparation of recombinant ADAMTS13 with a concentration much higher than normal plasma. Some laboratories did not take this into account when making assay dilutions and this was the main reason for dose-response slopes to differ from the reference preparations (samples L & A). In the majority of cases a valid comparison of dose-response slopes was possible, however, this was associated with considerable variability in estimates between laboratories. This could indicate that current methodologies are not optimised for testing the high purity recombinant material or that the proposed WHO 1st IS is not a suitable standard for the measurement of recombinant ADAMTS13. Estimates for antigen were approximately 4-times larger than estimates for function and the reason for this is unclear but it is possible that the recombinant preparation may contain inactive molecules and hence a reduced specific activity compared to ADAMTS13 in normal plasma.

Proposal

It is proposed that sample A/B (NIBSC code 12/252) is endorsed as the WHO 1st IS ADAMTS13 Plasma with assignment of the following consensus mean values:

Function: 0.91 IU per ampoule
Antigen: 0.92 IU per ampoule

Acknowledgements

The support of the participants listed in Appendix 1 is gratefully acknowledged for undertaking the testing in the collaborative study. We are also grateful to Baxter Innovations GmbH, Vienna, Austria for the supply of recombinant ADAMTS13 (Sample E); to Kainos Laboratories, Tokyo, Japan for providing test kits for the evaluation of candidate materials and to the chair and members of the SSC Sub-committee on von Willebrand factor for their support.

Instructions for Use

The draft Instructions for Use for the proposed WHO 1st IS ADAMTS13 Plasma are found in Appendix 4.

References

Campbell PJ. International biological standards and reference preparations. 1. Preparation and presentation of materials to serve as standards and reference preparations. J Biol Standardisation 1974; **2**: 249-67

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

Grubbs F. Procedures for detecting outlying observations in samples. Technometrics 1969; **11**: 1-21

Kirkwood TBL. Geometric means and measures of dispersion. Biometrics 1979; **35**: 908-9

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

Rieger M, Ferrari S, Kremer Hovinga JA, Konetschny C, Herzog A, Koller L, Weber A, Remuzzi G, Dockal M, Plaimauer B, Scheiflinger F. Relation between ADAMTS13 activity and ADAMTS13 antigen levels in healthy donors and patients with thrombotic microangiopathies (TMA). Thromb Haemost 2006; **95**: 212-20

Tripodi A, Chantarangkul V, Böhm M, Budde U, Fong J-F, Friedman KD, Galbusera M, Girma J-P, Moake J, Rick ME, Studt J-D, Turecek PL, Mannucci PM. Measurment of von Willebrand factor cleaving protease (ADAMTS-13): results of an international collaborative study involving 11 methods testing the same set of coded plasmas. J Thromb Haemost 2004; **2**: 1601-09

Tripodi A, Peyvandi F, Chantarangkul V, Palla R, Afrasiabi A, Canciani MT, Chung DW, Ferrari S, Fujimura Y, Karimi M, Kokame K, Kremer Hovinga JA, Lämmle B, De Meyer SF, Plaimauer B, Vanhoorelbeke K, Varadi K, Mannucci PM. Second international collaborative study evaluating performance characteristics of methods measuring the von Willebrand factor cleaving protease (ADAMTS-13). J Thromb Haemost 2008; **6**: 1534-41

TABLE 1 Estimates for ADAMTS13 function (units/ml) in the proposed WHO 1st IS ADAMTS13 Plasma (Samples A and B) relative to local plasma pools (Sample L)

Lab No.	Method	Sample A			Sample B		
		Mean	GCV%	n	Mean	GCV%	n
1	FRET	1.00	5.3	4	0.93	3.4	4
2	FRET	0.94	4.3	4	0.93	3.3	4
3	Residual VWF activity	1.00	14.9	4	1.01	11.1	4
4	Act ELISA	0.95	2.7	4	0.95	2.5	4
5	Gold Particle	0.92	1.2	4	0.92	1.4	4
6	Chemiluminescence	0.83	4.2	4	0.82	4.4	4
8*	FRET	1.10	8.6	4	1.02	7.4	4
10	Act ELISA	0.81	4.3	4	0.85	2.8	4
11	FRET	0.92	9.4	3	0.94	3.9	3
12a	FRET	0.86	9.7	4	0.81	5.8	4
12b	Act ELISA	0.85	1.5	2	0.84	0.7	2
12c	Collagen Binding	0.99	9.8	3	0.98	9.6	3
13	FRET	0.79	4.3	4	0.79	4.7	4
14	FRET	0.94	2.5	4	0.93	2.4	4
15*	Act ELISA	0.97	1.0	4	1.02	1.0	3
16a	Act ELISA	0.73	7.2	4	0.72	7.1	4
16b	FRET (Combi)	0.95	5.4	4	1.01	9.7	4
17	Act ELISA	0.97	8.9	3	0.89	17.6	3
18*	FRET	0.86	17.8	4	0.87	17.0	4
19	FRET	0.90	21.3	3	0.94	3.4	3
20	FRET	1.21	5.9	4	1.19	6.1	4
21	FRET	1.06	8.3	3	1.01	3.8	3
22	FRET	0.74	4.9	3	0.72	3.3	3
23	FRET	0.91	2.6	4	0.93	5.4	4
24	Residual RCo	0.88	1.5	4	0.87	2.0	4
26	Collagen Binding	1.21	--	1	1.13	--	1
28	FRET	0.83	9.5	4	0.84	8.3	4
30	Act ELISA	0.80	2.7	4	0.80	1.8	4
31	Act ELISA	0.84	23.6	3	0.85	12.2	3
32	FRET	0.86	0.6	4	0.87	4.9	4
33	Act ELISA	0.91	16.3	4	0.87	8.8	4
34	FRET	1.05	9.2	4	1.07	7.0	4
35	FRET	0.82	10.4	4	0.84	5.8	4
FRET Mean (n = 18)		0.92	GCV 13.2%		0.92	GCV 12.6%	
Activity ELISA Mean (n = 9)		0.87	GCV 10.4%		0.86	GCV 10.4%	
Overall Mean (n = 33)		0.91	GCV 12.9%		0.91	GCV 12.2%	
Combined Mean (Samples A & B)		0.91 GCV 12.4%					

Samples A and B are coded duplicates of the proposed WHO 1st IS ADAMTS13 Plasma (12/252)
*** - results based on participant's own calculations and normalised vs sample L**
n - number of individual assays included in analysis

TABLE 2 Estimates for ADAMTS13 function (units/ml) in Samples C, D and E relative to local plasma pools (Sample L)

Lab No.	Method	Sample C (Patient sample)		Sample D (Patient sample)		Sample E (Recombinant ADAMTS13)	
		Mean	GCV%	Mean	GCV%	Mean	GCV%
1	FRET	0.137	43.0	0.28	15.1	79.5	8.4
2	FRET	BQ	--	0.25	8.8	67.1	10.0
3	Residual VWF activity	0.008	88.1	0.10	12.6	71.7	22.8
4	Act ELISA	0.017	13.9	0.14	11.9	84.4	5.7
5	Gold Particle	0.031	1 assay	0.13	3.9	79.1	1.0
6	Chemiluminescence	0.024	6.9	0.15	3.8	68.1	4.5
8*	FRET	0.060	1 assay	0.18	30.2	56.3	46.7
10	Act ELISA	BQ	--	0.12	5.2	31.3	8.1
11	FRET	BQ	--	0.23	5.5	72.7	6.2
12a	FRET	0.033	24.8	0.17	9.0	68.5	3.1
12b	Act ELISA	BQ	--	0.13	4.2	42.5	2.9
12c	Collagen Binding	BQ	--	DR	--	57.6	5.7
13	FRET	BQ	--	0.17	7.4	58.4	10.2
14	FRET	0.056	5.2	0.22	4.6	74.8	2.0
15*	Act ELISA	BQ	--	DR	--	34.0	9.4
16a	Act ELISA	BQ	--	0.13	2.3	41.7	11.6
16b	FRET (Combi)	BQ	--	0.07	22.3	59.1	21.0
17	Act ELISA	0.020	77.1	0.21	42.9	21.4	48.0
19	FRET	BQ	--	DR	--	52.7	66.2
20	FRET	BQ	--	0.44	14.4	112.4	17.0
21	FRET	BQ	--	0.13	65.6	25.2	47.8
22	FRET	BQ	--	0.15	14.1	70.7	25.7
23	FRET	0.019	17.4	0.15	10.1	35.4	20.2
24	Residual RCo	BQ	--	DR	--	71.0	6.0
26	Collagen Binding	BQ	--	BQ	--	DR	--
28	FRET	BQ	--	BQ	--	57.8	8.4
30	Act ELISA	BQ	--	0.09	3.2	43.9	6.0
31	Act ELISA	BQ	--	0.15	49.9	46.2	1.4
32	FRET	0.015	30.2	0.15	6.1	75.0	5.0
33	Act ELISA	BQ	--	0.06	57.8	DR	--
34	FRET	BQ	--	BQ	--	77.2	10.9
35	FRET	BQ	--	DR	--	DR	--
FRET Mean		0.040 (n=6)	126.6%	0.18 (n=13)	55.0%	62.0 (n=16)	40.9%
Activity ELISA Mean		0.018 (n=2)	--	0.12 (n=8)	44.9%	40.3 (n=8)	47.8%
Overall Mean All Assays		0.028 (n=11)	120.2%	0.15 (n=24)	54.0%	56.1 (n=29)	47.0%

*** - results based on participant's calculations and normalised vs sample L**
BQ - no estimate possible, responses below the level for quantification relative to sample L
DR - no estimate possible, poor dose-response or dose-response out of range of sample L

TABLE 3 Estimates for ADAMTS13 function (units/ml) in Samples C, D and E relative to the proposed WHO 1st IS Plasma (Sample A) (assigned 0.91 units/ml)

Lab No.	Method	Sample C (Patient sample)		Sample D (Patient sample)		Sample E (Recombinant ADAMTS13)	
		Mean	GCV%	Mean	GCV%	Mean	GCV%
1	FRET	0.110	50.6	0.26	10.2	73.3	8.3
2	FRET	BQ	--	0.26	8.2	65.6	10.6
3	Residual VWF activity	0.011	35.2	0.09	33.9	63.1	35.3
4	Act ELISA	0.016	19.4	0.14	17.1	81.4	4.2
5	Gold Particle	0.031	1 assay	0.13	2.1	77.4	1.6
6	Chemiluminescence	0.027	3.7	0.16	4.7	75.1	2.5
8*	FRET	0.06	1 assay	0.18	30.23	56.3	46.5
10	Act ELISA	BQ	--	0.14	9.9	33.6	11.7
11	FRET	BQ	--	0.24	3.0	73.5	6.3
12a	FRET	0.039	10.5	0.19	8.9	72.0	7.3
12b	Act ELISA	BQ	--	0.15	2.5	47.4	2.5
12c	Collagen Binding	BQ	--	DR	--	50.4	18.8
13	FRET	BQ	--	0.13	11.0	57.7	5.4
14	FRET	0.051	11.0	0.21	2.6	73.1	2.0
15*	Act ELISA	BQ	--	DR	--	34.0	9.4
16a	Act ELISA	BQ	--	0.10	10.7	32.1	18.3
16b	FRET (Combi)	BQ	--	0.04	21.5	35.1	18.3
17	Act ELISA	0.028	51.9	0.24	17.8	25.5	4.1
19	FRET	BQ	--	DR	--	57.8	47.1
20	FRET	BQ	--	0.32	5.1	83.5	16.1
21	FRET	BQ	--	0.14	47.9	26.2	33.2
22	FRET	BQ	--	0.22	9.0	93.4	22.6
23	FRET	0.019	25.8	0.15	11.4	36.2	23.8
24	Residual RCo	BQ	--	DR	--	73.7	5.3
26	Collagen Binding	BQ	--	BQ	--	DR	--
28	FRET	BQ	--	BQ	--	60.3	16.6
30	Act ELISA	BQ	--	0.11	5.4	49.6	4.9
31	Act ELISA	BQ	--	0.17	30.7	44.7	8.5
32	FRET	0.017	24.4	0.16	5.6	78.3	5.3
33	Act ELISA	BQ	--	0.05	41.9	DR	--
34	FRET	BQ	--	BQ	--	64.8	9.0
35	FRET	BQ	--	DR	--	DR	--
FRET Mean		0.040 (n=6)	103.8%	0.17 (n=13)	67.8%	59.9 (n=16)	41.1%
Activity ELISA Mean		0.022 (n=2)	--	0.13 (n=8)	58.5%	41.0 (n=8)	43.0%
Overall Mean All Assays		0.030 (n=11)	94.7%	0.15 (n=24)	63.2%	55.0 (n=29)	44.6%
**Overall Mean Assays exc. slopes outside 80-125%		0.029 (n=11)	84.6%	0.15 (n=21)	63.2%	57.7 (n=25)	39.1%

*** - results based on participant's calculations and normalised vs sample L**
**** - excluding assays where the dose-response ratio with sample A is outside 80 - 125%**
BQ - no estimate possible, responses below the level for quantification relative to sample A
DR - no estimate possible, poor dose-response or dose-response out of range of sample A

TABLE 4 Estimates for ADAMTS13 antigen (units/ml) in proposed WHO 1st IS ADAMTS13 Plasma (Samples A and B) relative to local plasma pools (Sample L)

Lab No.	Sample A			Sample B		
	Mean	GCV%	n	Mean	GCV%	n
1	0.80	9.2	3	0.82	4.9	3
2	0.94	18.3	3	0.93	16.6	2
4	0.73	1.9	4	0.64	1.4	4
8*	1.17	--	1	1.07	--	1
10	1.07	5.0	4	1.09	3.5	4
12	0.90	6.1	4	0.91	9.1	4
13	0.86	--	1	0.91	--	1
15	0.93	4.8	4	0.90	3.1	4
16a	1.08	8.4	4	1.13	12.8	4
16b	1.06	10.1	4	1.04	3.7	4
19	0.78	4.2	4	0.78	6.0	4
21	1.08	16.0	3	1.09	1.0	3
23	0.98	4.4	2	0.93	5.9	2
28	0.72	12.3	4	0.74	7.7	4
31	1.01	21.6	3	1.00	15.8	3
32	0.85	3.5	4	0.85	4.0	4
Overall Mean (n = 16)	0.93	GCV 16.2%		0.92	GCV 17.0%	
Combined Mean (A & B)	0.92 GCV 16.3%					

Samples A and B are coded duplicates of the proposed WHO 1st IS ADAMTS13 Plasma (12/252)
*** - results based on participant's own calculations and normalised vs sample L**
n - number of individual assays included in analysis

TABLE 5 Estimates for ADAMTS13 antigen (units/ml) in Samples C, D and E relative to local plasma pools (Sample L)

Lab No.	Sample C (Patient sample)		Sample D (Patient sample)		Sample E (Recombinant ADAMTS13)	
	Mean	GCV%	Mean	GCV%	Mean	GCV%
1	0.24	15.8	0.75	16.7	404	8.0
2	0.24	17.2	0.55	16.4	63	124.8
4	0.12	3.8	0.27	6.4	382	2.5
8*	0.21	1 assay	0.55	1 assay	180	1 assay
10	0.36	8.5	0.98	4.8	437	14.4
12	0.21	2.9	0.44	10.6	124	7.0
13	0.42	17.3	0.73	12.8	149	9.8
15	0.33	2.8	0.80	4.8	274	4.1
16a	0.27	7.5	0.93	5.1	380	8.9
16b	0.30	4.2	0.93	4.9	454	27.1
19	0.25	25.0	0.50	24.1	123	50.0
21	DR	--	1.00	2.5	306	54.2
23	BQ	--	BQ	--	225	13.0
28	0.22	11.7	0.47	13.1	149	15.0
31	0.29	47.1	0.71	14.9	256	28.2
32	0.21	1.1	0.44	1.8	150	2.1
Overall Mean All Assays	0.25 (n=14)	35.3%	0.63 (n=15)	45.8%	221 (n=16)	76.8%

*** - results based on participant's own calculations and normalised vs sample L**
BQ - no estimate possible, responses below the level for quantification relative to sample L
DR - no estimate possible, poor dose-response or dose-response out of range of sample L

TABLE 6 Estimates for ADAMTS13 antigen (units/ml) in Samples C, D and E relative to the proposed WHO 1st IS ADAMTS13 Plasma (Sample A) (assigned 0.92 units/ml)

Lab No.	Sample C (Patient sample)		Sample D (Patient sample)		Sample E (Recombinant ADAMTS13)	
	Mean	GCV%	Mean	GCV%	Mean	GCV%
1	0.29	11.3	0.82	10.6	445	5.8
2	0.22	9.5	0.55	2.6	60	120.1
4	0.15	3.3	0.33	5.9	470	3.8
8*	0.21	1 assay	0.55	1 assay	180	1 assay
10	0.31	8.0	0.83	3.9	370	10.6
12	0.21	2.8	0.44	9.0	125	3.0
13	0.47	16.8	0.77	15.2	156	11.4
15	0.32	7.0	0.78	5.2	299	3.4
16a	0.23	9.0	0.77	5.3	329	2.7
16b	0.26	14.3	0.79	5.4	392	35.5
19	0.29	19.9	0.60	20.9	148	49.2
21	DR	-	0.81	19.9	257	62.2
23	BQ	-	BQ	-	235	6.9
28	0.29	15.1	0.60	5.4	193	13.7
31	0.30	16.0	0.69	22.2	220	63.5
32	0.22	3.1	0.46	4.4	163	3.0
Overall Mean All Assays	0.26 (n=14)	30.9%	0.63 (n=15)	32.0%	224 (n=16)	71.7%
**Overall Mean exc. slopes outside 80-125%	0.26 (n=14)	30.1%	0.64 (n=14)	30.3%	222 (n=13)	83.1%

*** - results based on participant's own calculations and normalised vs sample L**
**** - excluding assays where the dose-response ratio with sample A is outside 80 - 125%**
BQ - no estimate possible, responses below the level for quantification relative to sample A
DR - no estimate possible, poor dose-response or dose-response out of range of sample A

TABLE 7 Ratio of ADAMTS13 function to antigen

Mean estimate	Sample						
	A/B	C		D		E	
	vs L	vs A	vs L	vs A	vs L	vs A	vs L
Function (units/ml)	0.91	0.03	0.028	0.15	0.15	55.0	56.1
Antigen (units/ml)	0.92	0.26	0.25	0.63	0.63	224	221
Ratio function/antigen	**0.99**	**0.12**	**0.11**	**0.24**	**0.24**	**0.25**	**0.25**

TABLE 8 Accelerated degradation study of the proposed WHO 1st IS (12/252). Mean residual potencies of ampoules stored at elevated temperatures for 10 months expressed relative to ampoules stored at -70 °C.

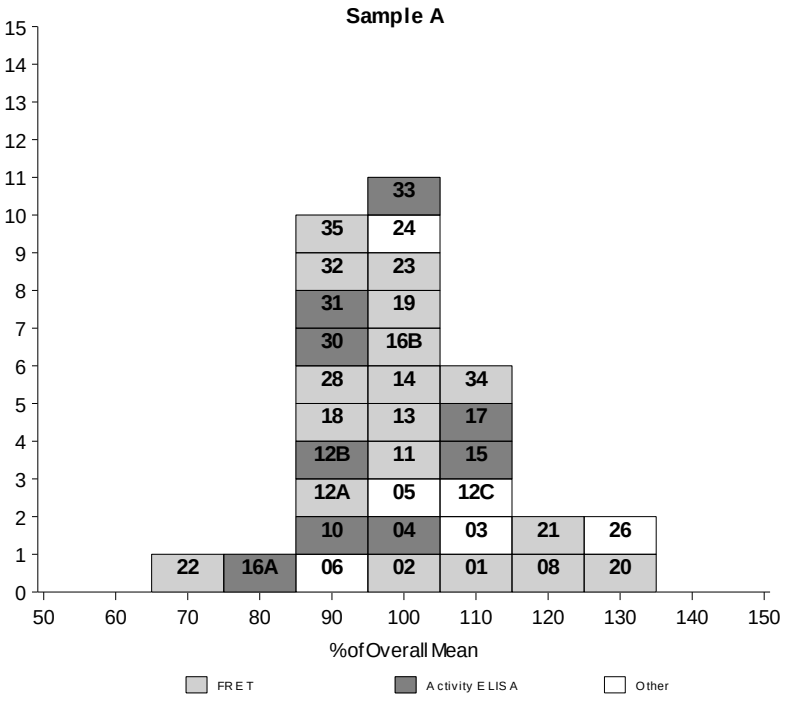
Analyte	Storage Temperature (°C)				
	-20	+4	+20	+37	+45
Function: Activity ELISA	100%	98%	98%	90%	70%
Antigen: ELISA	98%	98%	95%	69%	50%

Results are the means of 4 independent assays

TABLE 9 Accelerated degradation study of the proposed WHO 1st IS (12/252). Predicted degradation rates for ampoules at various storage temperatures expressed as % loss per year.

Analyte	Storage Temperature (°C)		
	-20	+4	+20
Function	**0.001%**	0.07%	1.14%
Antigen	**0.06%**	1.29%	7.40%

Figure 1 Mean laboratory estimates for ADAMTS13 function in the proposed WHO 1st IS Plasma 12/252 (samples A and B) relative to the local pooled normal plasma (sample L) as percentage of the overall mean value



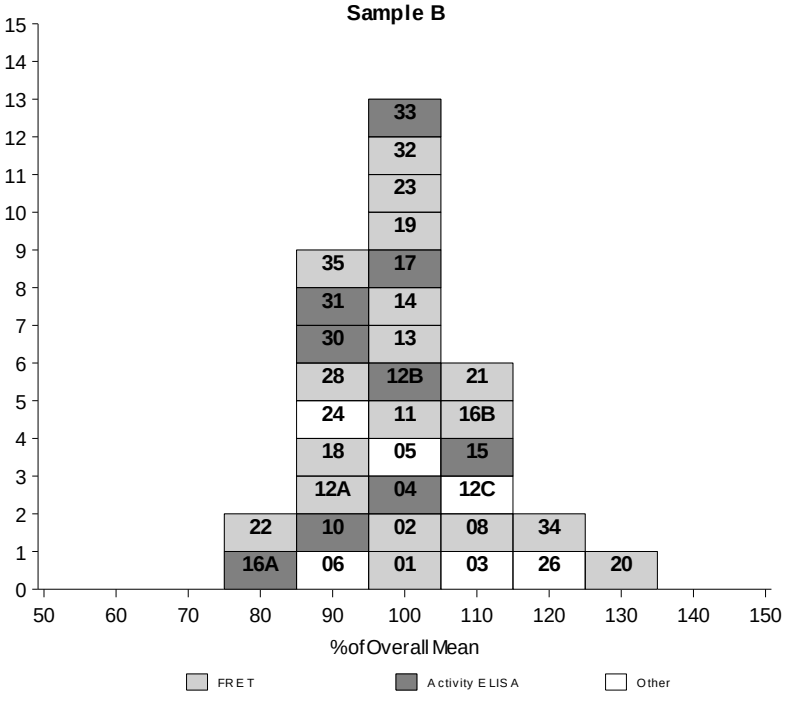
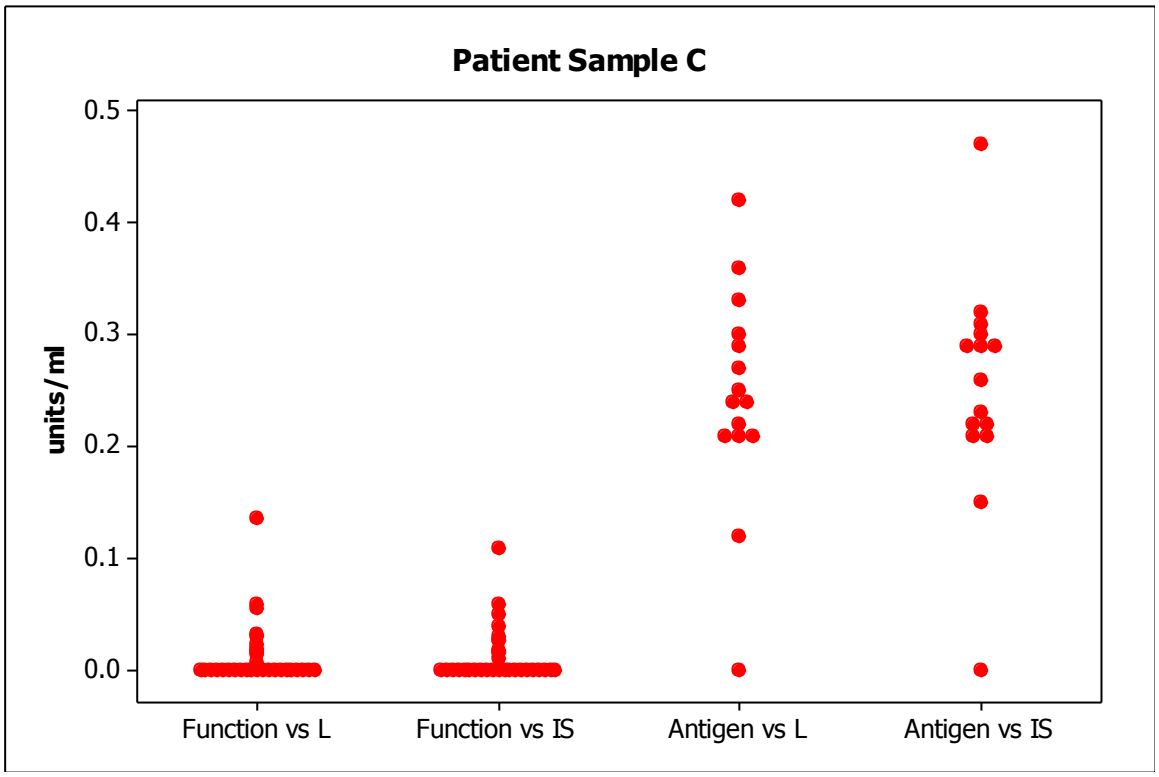
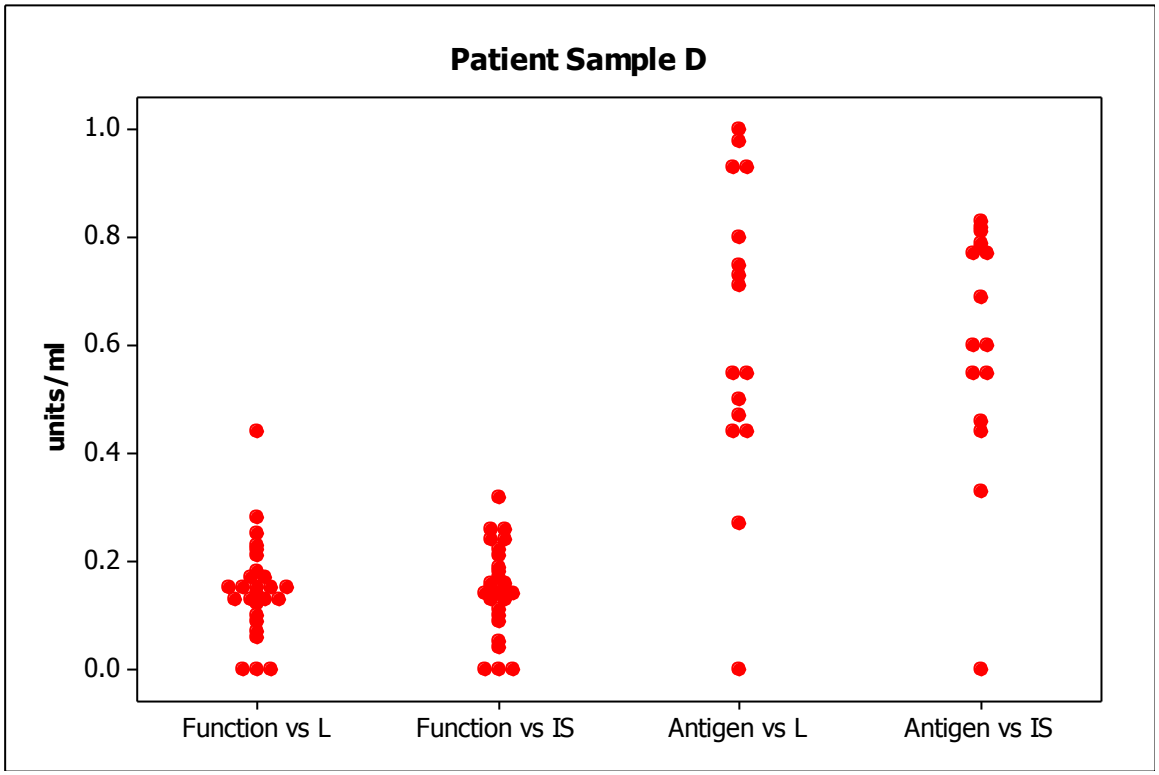


Figure 2 Mean laboratory estimates for ADAMTS13 function and antigen in Sample C (patient plasma) relative to the local pooled normal plasma (sample L) and the proposed WHO 1st IS ADAMTS13 Plasma (12/252) (sample A)



Laboratory estimates below the level of quantification are plotted as zero

Figure 3 Mean laboratory estimates for ADAMTS13 function and antigen in Sample D (patient plasma) relative to the local pooled normal plasma (sample L) and the proposed WHO 1st IS ADAMTS13 Plasma (12/252) (sample A)



Laboratory estimates below the level of quantification are plotted as zero

Figure 4 Mean laboratory estimates for ADAMTS13 function and antigen in Recombinant ADAMTS13 (sample E) relative to the local pooled normal plasma (sample L) and the proposed WHO 1st IS ADAMTS13 Plasma (12/252) (sample A)

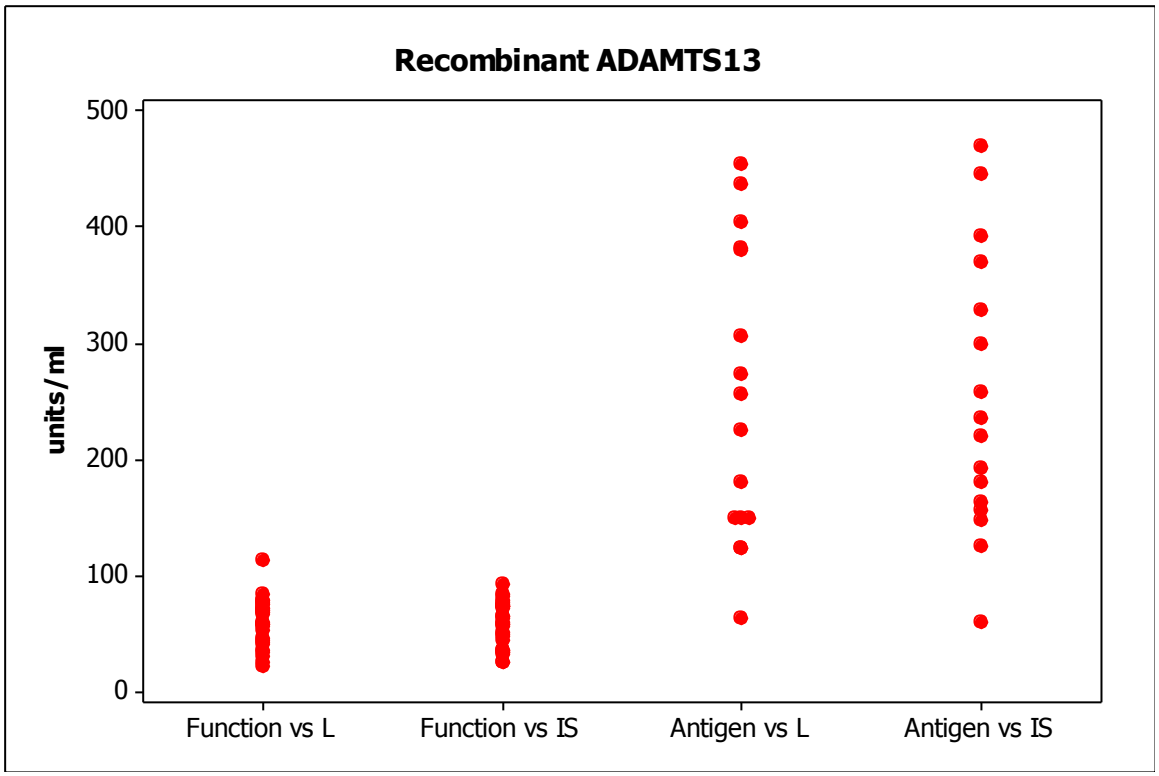
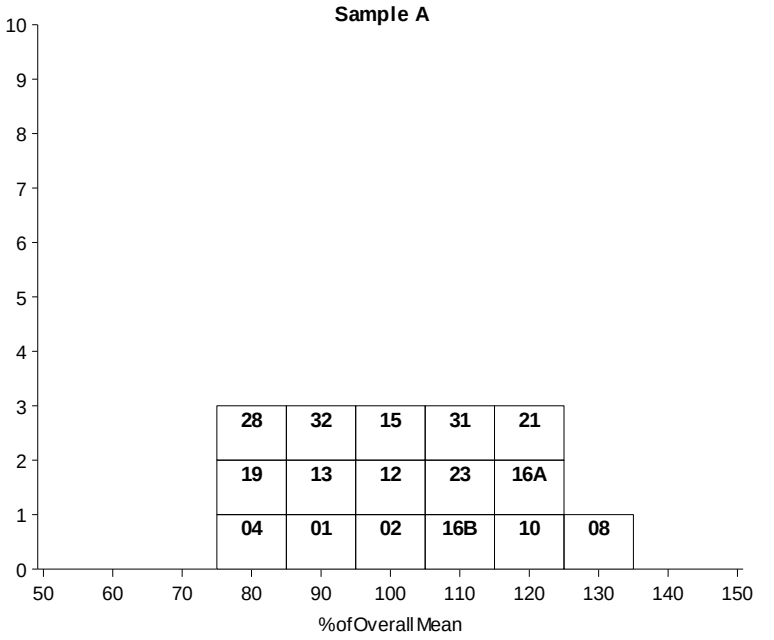
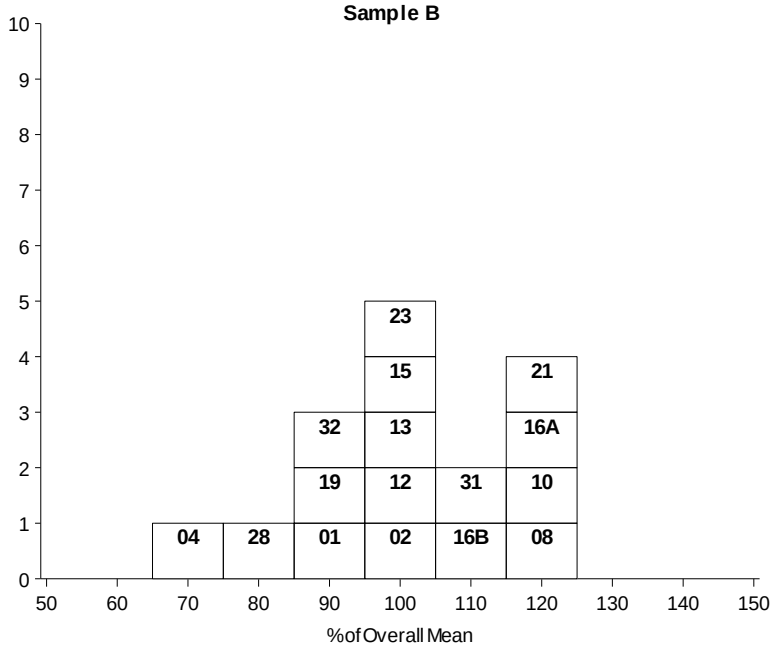


Figure 5 Mean laboratory estimates for ADAMTS13 antigen in the proposed WHO 1st IS Plasma 12/252 (samples A and B) relative to the local pooled normal plasma (sample L) as percentage of the overall mean value





Appendix 1 List of Participants in the Collaborative Study

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Dr E Grandone / Dr G Tiscia, Casa Sollievo della Sofferenza Institute, Atherosclerosis and Thrombosis Unit, San Giovanni Rotondo (FG), Italy

Prof U Budde, Hämostaseology, Asklepios Klinik Altona, Hamburg, Germany

Dr H Rottensteiner, Baxter Innovations GmbH, Vienna, Austria

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Dr S Kitchen / Ms RE Fretwell, Coagulation Dept., Royal Hallamshire Hospital, Sheffield, UK

Dr SC Nair, Div. Laboratory Haematology, Christian Medical College, Vellore, Tamil Nadu, India

Prof K Vanhoorelbeke, Lab. for Thrombosis Research, IRF Life Sciences, Kortrijk, Belgium

Appendix 2 Study Protocol

Primary objective of the study

Value assignment of the candidate WHO 1st International Standard (IS) (12/252) for ADAMTS13 function and antigen in units relating to plasma equivalents (where 1 unit/ml = 100% normal) by assay relative to local pooled normal plasma preparations. Kit standards should only be used if no local pooled normal plasma is available.

1 Samples included in the assays

Samples A to E are provided by NIBSC. **Sample L** is provided by each participating laboratory and is your local pooled normal plasma preparation - this will be used to calculate the potencies of samples A to E. Laboratories measuring both function and antigen will receive 8 ampoules/vials of samples A to E. Laboratories using only one method will receive 4 ampoules/vials of samples A to E.

A - Proposed WHO 1st IS ADAMTS13, Plasma (12/252) - pooled normal plasma
B - A second pooled normal plasma
C - Patient Sample -1 - "waste" from plasma exchange of acquired deficiency
D - Patient Sample -2 - "waste" from plasma exchange of acquired deficiency
E - Purified ADAMTS13
L - Your local pooled normal plasma preparation with a local assigned value or an arbitrary value of 100% or 1.0 unit per ml.

2 Storage and reconstitution of samples

Samples A to D - Store unopened ampoules at -20°C or below
Allow the ampoules to warm to room temperature before reconstitution. Tap ampoules gently to ensure that all of the contents are in the lower part of the container. Ensure the disposable ampoule safety breaker is pushed down on the stem of the ampoule against the shoulder of the ampoule body. Apply a bending force to open the ampoule at the coloured stress point. Care should be taken to avoid cuts and projectile glass fragments. Reconstitute by adding **1 ml** of distilled water at room temperature. Dissolve the contents with gentle agitation at room temperature. When reconstitution is complete transfer the entire contents to a plastic tube and proceed with the assay as soon as possible.

Sample E - Store unopened vials at 2 to 8°C
Allow the vials to warm to room temperature before reconstitution. Tap vials gently to ensure that all of the contents are in the lower part of the vial. Carefully remove the aluminium crimping cap and the rubber stopper to release the vacuum. Slowly add **5 ml** of distilled water (at room temperature) along the wall of the vial and move the pipette to ensure that the whole lyophilisate is covered. Re-insert the rubber stopper. Leave for 1 minute at room temperature and swirl carefully to aid reconstitution which should be complete in less than 10 minutes. When reconstitution is complete transfer the contents to a plastic tube and begin testing as soon as possible.

3 General plan of the study

You are requested to carry out 4 assays by each method using fresh ampoules/vials for each assay. The 4 assays should be spread over at least 2 separate days/sessions. Sufficient ampoules have been provided for freshly reconstituted ampoules to be used for each assay. If you are performing more than 2 methods then you will have to use the same ampoules for more than one method. Please let me know if you require more ampoules.

Assays for ADAMTS13 function must be carried out on freshly reconstituted ampoules and vials of A to E.

Assays for ADAMTS13 antigen should also be carried out on freshly reconstituted ampoules but may be carried out on frozen aliquots if this is unavoidable. Please indicate in your results if frozen aliquots have been used.

4 Assay Dilutions

The approximate content of ADAMTS13 in "plasma equivalent units" (where 1 unit/ml = 100% normal) for samples A to E after reconstitution is given in the table below as an aid to preparing your assay dilutions. Each assay must include 3 or more different dilutions of all samples (eg. 1/50, 1/100, 1/200), including your local pooled normal plasma (L), in order that the dose-responses of all samples can be compared. **It may not be possible to obtain a dose-response for Sample C because of the low ADAMTS13 content - in this case the testing of a single dilution is acceptable.** You may need to adjust your assay dilutions based on the results of your first assay in order to obtain a better overlap of responses - please give priority to Samples A, B and L which are of primary importance in the study.

Table ADAMTS13 concentration of samples after reconstitution

Sample	Reconstitution volume (ml)	ADAMTS13 concentration after reconstitution (units/ml)
A	1.0	0.8 - 1.0
B	1.0	0.8 - 1.0
C	1.0	0.1 - 0.2
D	1.0	0.2 - 0.4
E	5.0	50 - 70

5 Assay Design

All five samples (A to E) and your local pooled normal plasma (L) should be included in each of the 4 assays. A minimum of 3 dilutions of each preparation should be tested (with the possible exception of Sample C), in replicate, within each assay. Please use your normal testing methodology but follow a balanced assay design such as described below. In the following design, each letter refers to a separate set of dilutions and A, A' and B, B' etc. refer to fresh sets of dilutions (replicates) made from the same ampoule/vial.

Assay Design

Assay 1	L	A	B	C	D	E	E'	D’	C’	B’	A’	L'
Assay 2	E	D	C	B	A	L	L'	A’	B’	C’	D’	E'
Assay 3	L	C	D	E	B	A	A'	B’	E’	D’	C’	L'
Assay 4	D	E	A	B	C	L	L'	C'	B’	A’	E’	D’

6 Remaining samples

As agreed on the invitation to the study, it is understood that all remaining samples of A to E will be destroyed after this testing is complete.

7 Results

Please return the raw data from your assays and also your calculated potencies and methodology details using the Excel results sheets **by 15 November 2013** to: **anthony.hubbard@nibsc.org**
Please ensure that your raw data is returned as optical density etc. rather than as % or units relative to a local reference and remember to give details of all dilution steps (pre-dilution and working dilutions) in the results sheets.

Appendix 3 Details of Methods and Local Pooled Normal Plasma

Lab No	ADAMTS13 Function	ADAMTS13 Antigen	Local Pooled Normal Plasma	
			No. Donors	Assigned value
1	FRET	Technozym kit	42	100% , 1 u/ml
2	FRET	Imubind kit	6	100%
3	Residual VWF Activity	----	20	NG
4	Activity ELISA	In house	50	100%, 1 u/ml
5	Gold Particle VWF-73	----	50	1 u/ml
6	Chemiluminescence VWF-73	----	285	100%, 1 u/ml
8	FRET	Imubind kit	NG	
9	FRET VWF-86	Imubind kit	30	100%
10	Activity ELISA	Technozym kit	50	100%, 1 u/ml
11	FRET	----	60	100%, 1 u/ml
12	a) FRET b) Activity ELISA c) Collagen Binding	In house	79 79 64	1 u/ml 1 u/ml 1 u/ml
13	FRET	In house	90	100%, 1 u/ml
14	FRET	----	>20	94%
15	Activity ELISA	Technozym kit	>20	1 u/ml activity 0.76 u/ml antigen
16	a) Activity ELISA b) FRET (Combi-Actibind)	a) Combi-Actibind kit b) Technozym kit	NG	61.75% activity 76% antigen
17	Activity ELISA	----	25	100%
18	FRET	----	50	1.27 iu/ml
19	FRET	In house	20	100%
20	FRET VWF-71	----	35	1 u/ml
21	FRET	Technozym kit	>30	1 u/ml
22	FRET	----	20	100%
23	FRET	In house	50	1 u/ml
24	Residual VWF:RCo Activity	----	250	1 u/ml
25	Activity ELISA	Technozym kit	NG	1 u/ml
26	Collagen Binding	----	NG	0.99 u/ml
28	FRET	Imubind kit	40	100%, 1 u/ml
30	Activity ELISA	----	56	1 u/ml
31	Activity ELISA	Technozym kit	NG	1 u/ml
32	FRET	In house	73	1 u/ml
33	Activity ELISA	----	32	100%
34	FRET	----	20	1 u/ml
35	FRET XS-VWF	----	5	1 u/ml

NOTES:
All FRET and Activity ELISA methods used the VWF-73 peptide unless indicated otherwise. The VWF-73 peptide was purchased from the following sources either as a separate reagent or as a kit component: Peptide International, Peptanova, Peptide Institute, Kainos Labs., Technoclone. NG - not given

Appendix 4

Draft Instructions for Use for the proposed WHO 1st International Standard ADAMTS13 Plasma

WHO International Standard
WHO 1st International Standard ADAMTS13 Plasma
NIBSC code: 12/252
Instructions for use
(Version 1.00, Dated)

1. INTENDED USE

The WHO 1st International Standard for ADAMTS13 in plasma was established by the Expert Committee on Biological Standardisation of the World Health Organisation in 2014 and details of the preparation and value assignment are available in document WHO/BS/14.***. The preparation consists of glass ampoules (coded 12/252) containing 1 ml aliquots of pooled normal human plasma, freeze-dried. The International Standard (IS) has values assigned for ADAMTS13 function and antigen.
The standard is intended to be used for the estimation of these analytes in human plasma.

2. CAUTION

This preparation is not for administration to humans.

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 assigned values were determined by comparison relative to locally collected pooled normal human plasma in an international collaborative study involving 32 laboratories in 14 countries. The overall mean values assigned to each ampoule of the WHO 1st IS are as follows:

ADAMTS13 function	0.91 IU per ampoule
ADAMTS13 antigen	0.92 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 +/- 0.245%.

4. CONTENTS

Country of origin of biological material: United Kingdom.
The WHO 1st IS was prepared at the National Institute for Biological Standards and Control in February 2013 from a pool of 10.2 litres of plasma collected from 38 normal healthy donors. Blood was collected by conventional venepuncture into citrate-phosphate-dextrose-adenine anticoagulant. Each donation underwent leuco-filtration before being centrifuged twice to remove all cellular components. The units were then frozen and stored at -70°C. Plasma was thawed on the day of filling, pooled and then buffered by the addition of HEPES (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]) to a final concentration of 40 mmol/l. One ml of the pooled plasma was dispensed into each of approximately 10,200 ampoules. Freeze-drying was performed in accordance with the conditions required for International Standards (1). The mean liquid filling weight was 1.0079g and the coefficient of variation was 0.245% based on 420 check-weight ampoules. Mean residual moisture after freeze-drying was 0.35% (n=12). Mean oxygen concentration in the headspace was 0.29% (n=12).

5. STORAGE

Unopened ampoules should be stored in the dark at -20°C or below.
Please note: because of the inherent stability of lyophilized material, NIBSC may ship these materials at ambient temperature.

6. DIRECTIONS FOR OPENING

DIN ampoules have an 'easy-open' coloured stress point, where the narrow ampoule stem joins the wider ampoule body.
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

No attempt should be made to weigh out any portion of the freeze-dried material prior to reconstitution
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. Studies have shown the reconstituted standard to be stable for up to 4 hours when kept on melting ice, however, it is recommended that assays be carried out as soon as possible after reconstitution is complete. It is not recommended that frozen aliquots are used.

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.

9. REFERENCES

1. Campbell PJ. Procedures 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 Centre for Biological Reference Materials (NIBSC) and to the chair and members of the SSC/ISTH sub-committee for von Willebrand factor for their support.

11. FURTHER INFORMATION

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

12. CUSTOMER FEEDBACK

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

13. CITATION

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

14. MATERIAL SAFETY SHEET

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 ampoule contents should be taken up with absorbent material wetted with an appropriate disinfectant. Rinse area with an appropriate disinfectant followed by water. Absorbent materials used to treat spillage should be treated as biological waste.

15. LIABILITY AND LOSS

In the event that this document is translated into another language, the English language version shall prevail in the event of any inconsistencies between the documents.

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

16. INFORMATION FOR CUSTOMS USE ONLY

Country of origin for customs purposes*: United Kingdom * Defined as the country where the goods have been produced and/or sufficiently processed to be classed as originating from the country of supply, for example a change of state such as freeze-drying.
Net weight: 0.079 g
Toxicity Statement: Non-toxic
Veterinary certificate or other statement if applicable. **Attached**: No

17. CERTIFICATE OF ANALYSIS

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