World Health Organization

WHO/BS/2019.2370
ENGLISH ONLY

EXPERT COMMITTEE ON BIOLOGICAL STANDARDIZATION
Geneva, 21 to 25 October 2019

A Collaborative Study to Additionally Assign Value for Total Factor XIII-B Subunit Antigen to the WHO 1st International Standard for Factor XIII Plasma, (02/206)

Sanj Raut[1§], Éva Katona[2], Carmen Coxon[1], Andrew Riches-Duit[1], László Muszbek[2], Verena Schroeder[3] & Peter Rigsby[1]

[1]National Institute for Biological Standards and Control, South Mimms, Potters Bar, Hertfordshire, EN6 3QG, UK;
[2]Department of Laboratory Medicine, University of Debrecen, 4032 Debrecen, Hungary;
[3]Department for BioMedical Research, University of Bern, 3008 Bern, Switzerland

[§]Principal investigator and coordinator of project

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 **27 September 2019** 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 Ivana Knezevic** at email: knezevici@who.int.

Summary

A collaborative study was undertaken to value assign the current WHO 1st International Standard (IS) Factor XIII (FXIII) Plasma for Total FXIII-B subunit, relative to locally collected normal plasma pools.

Laboratories were instructed to use a validated method (specific ELISA antibodies provided) for assessment of Total FXIII-B subunit antigen potency. All laboratories used this method with one laboratory using an additional in-house method also. Nine (9) data sets were received from 7 laboratories (37 assays in total), which provided a total of 35 valid estimates for this new assignment. Total FXIII-B subunit estimates were calculated relative to locally collected normal plasma pools, using an arbitrary value of 1.00 unit of Total FXIII-B subunit per ml, for each pool.

Combination of results produced an overall mean of 0.98 units/ml with an inter-laboratory variability (GCV%) of 18.3% [95% confidence interval: 0.86 - 1.11].

Proposal

It is proposed that the current WHO 1st International Standard (IS) Factor XIII (FXIII) Plasma (02/206) be additionally assigned with a Total FXIII-B subunit antigen potency of 0.98 IU/ml.

Introduction

The current WHO 1st IS Factor XIII, plasma (02/206)[1,2] was established in 2004 with an assigned potency for activity of 0.91 IU/ml and an assigned potency for antigen (A2B2 complex) of 0.93 IU/ml. This standard is currently used for measurement of FXIII (FXIII-A2B2 complex) potency (both activity and antigen) in patient's plasma for diagnosis of FXIII deficiencies and also in FXIII therapeutic concentrates.

Factor XIII (FXIII) circulates in plasma as a heterotetramer of two A and two B subunits (FXIII-A2B2). The two subunits are synthesised at different locations: FXIII-A is synthesised in cells of bone marrow origin, while FXIII-B is synthesised in hepatocytes. FXIII-A is activated by thrombin to form a transglutaminase that crosslinks fibrin fibres, antifibrinolytic proteins, and proteins of the extracellular matrix. FXIII-B acts as carrier protein for FXIII-A in plasma, and without FXIII-B the plasma half-life of FXIII-A is significantly reduced. In plasma, the two subunits form 1:1 complex. However, FXIII-B is in excess over FXIII-A with approximately 50% of total FXIII-B existing in complex with FXIII-A and approximately 50% of total FXIII-B existing in free form, while 99% of FXIII-A subunits are in complex.

FXIII-B subunit measurements are required for the diagnosis and characterization of the type of FXIII deficiency. Furthermore, therapy for FXIII-A deficiency with rFXIII-A relies on available FXIII-B. As, such, the proposal to additionally value assign the WHO 1st IS Factor XIII plasma (02/206) for Total FXIII-B subunit antigen was reviewed and endorsed by WHO/ECBS in 2016. Furthermore, following a feasibility study carried out in 2018, the proposal to additionally value assign the WHO 1st IS Factor XIII plasma (02/206) for Total FXIII-B subunit antigen was agreed at the ISTH/SSC Factor XIII and Fibrinogen Sub-Committee meeting in July 2018.

The primary objective of the study was to value assign the WHO 1st IS Factor XIII plasma (02/206) for Total FXIII-B subunit antigen based on assays relative to local normal plasma pools. This report describes the findings of the study.

The International Unit (IU) for Total FXIII-B Subunit Antigen
The IU for FXIII activity (and A2B2 antigen) was originally derived from the consensus mean of laboratories testing the WHO 1st IS FXIII plasma (02/206) relative to local normal plasma pools which were arbitrarily assigned a value of 1.00 IU/ml. The IU therefore approximates to the mean normal value in the population. Establishment of an IU for the new analyte (Total FXIII-B subunit antigen) will follow the same approach used for FXIII activity (and A2B2 antigen) and will rely on the consensus mean from assays relative to local normal plasma pools, which will each be arbitrarily assigned a value of 1.00 unit/ml.)

Participants

Study samples and protocols were dispatched to nine (9) laboratories, who had agreed to participate in the study. Seven (7) laboratories (from 6 different countries) completed the study and returned data for analysis. They are listed in Appendix A. The participants included 5

academic institutes and 2 national control authorities. Laboratories were coded for the study and the order of listing in Appendix A does not necessarily correspond with the numerical codes. All raw data returned by the participants were centrally analysed at NIBSC

Materials

The following **sample and reagents** were provided for the study:

Label	Description	NIBSC Code
1st IS	WHO 1st IS FXIII Plasma (1st IS)	**02/206**
*MAb1	MAb1 (Biotinylated Anti-Total FXIII-B monoclonal antibody)	-
*MAb3	MAb3 (HRP-Labelled Anti-Total FXIII-B monoclonal antibody)	-

*Total FXIII B-subunit antigen assay kit monoclonal antibodies were kindly donated by Dr Katona & Professor Muszbek, Debrecen, Hungary)

WHO 1st IS FXIII Plasma (02/206)

- assigned potency for activity of 0.91 IU/ml and
- assigned potency for antigen (A2B2 complex) of 0.93 IU/ml.

Total FXIII B-subunit - ELISA antigen assay kit reagents

In addition, ELISA kit monoclonal antibodies specific for Total FXIII-B Subunit Antigen (kindly provided by Dr Katona, Professor Muszbek, Debrecen, Hungary) were included with the samples:

- Capture antibody (MAb1) - Biotinylated anti-FXIII-B mouse monoclonal antibody, lyophilized.
- Detection antibody (MAb3) - HRP-conjugated anti-FXIII-B mouse monoclonal antibody, 1 mL in stabilizing solution.

Capture and detection antibodies are directed against different epitopes on FXIII-B subunit.

Assay methods and study design

Details of the assay methods and study design are given in Appendix B. All laboratories used the recommended ELISA specific for Total FXIII-B subunit. This is a sandwich enzyme immunoassay using monoclonal antibodies (provided to all participants) specific to different

epitopes on the FXIII-B subunit[3]. The sample dilution containing FXIII, peroxidase-labeled tag antibody against FXIII-B subunit (MAb3) and biotinylated capture antibody directed against a different epitope on the FXIII-B subunit (MAb1) were incubated in the wells of the streptavidin coated ELISA microtitre plate. The formed complex was firmly attached to the streptavidin coated surface through the biotin moiety of anti-FXIII-B (MAb1). Non-binding proteins were washed away and the peroxidase activity of anti-FXIII-B (MAb3) remaining with the complex was measured by adding TMB substrate (ready for use). After stopping the reaction by the addition of 2M H_2SO_4 the intensity of the colour produced was measured by an ELISA plate reader.

Participants were requested to carry out four (4) independent assays using the recommended ELISA method for Total FXIII-B subunit antigen, preferably over 2 separate days (Normal Plasma Pool NP_1 - Day 1; Normal Plasma Pool NP_2 - Day 2), rather than all on the same day. Sufficient ampoules of the WHO 1st IS FXIII Plasma (02/206) were provided to allow freshly reconstituted samples to be used in all assays. Raw assay data were returned to NIBSC for centralised analysis.

Statistical analysis

Relative potencies of test samples were calculated using parallel line analysis with a log transformation of OD values, using a minimum of three sample dilutions on the linear section of the dose-response curve[4]. In some assays it was necessary to exclude responses from single dilutions at the extreme ends of the dose-response to improve the linearity. Calculations were performed using the software CombiStats (v5.0 EDQM, Council of Europe). Non-linearity and non-parallelism of dose-response relationships were considered in the assessment of assay validity. All dose-response lines showing no significant non-linearity or non-parallelism ($p>0.01$) were accepted for further analysis. All instances of significant non-linearity ($p<0.01$) were assessed visually and accepted if the correlation (r value) exceeded 0.99. All instances of significant non-parallelism ($p<0.01$) were further assessed by calculation of the ratio of fitted slopes for the test and reference sample and accepted if the slope ratio was within 0.80 - 1.25.

Variability and combination of estimates

Relative potency estimates from all valid assays were combined to generate an unweighted geometric mean (GM) for each laboratory and these laboratory means were used to calculate overall unweighted geometric means for each sample. Where a laboratory performed more than one assay method (e.g. in-house method) or used different type of normal plasma pool (e.g. Fresh & Frozen), the results for each method/plasma pool type used were analysed as if from separate laboratories. Variability between assays (within laboratories) and between laboratories has been expressed using percentage geometric coefficients of variation (GCV%)[5]. Within-assay variation and between-assay variation (intra-laboratory variability) were also assessed as 95% confidence limits (see TABLE 2). Comparisons between test data (e.g. assays using fresh vs frozen normal plasma pools) were made by two-tailed t-test of log transformed laboratory mean estimates. The mean laboratory estimates were assessed for outlying values using a Grubb's outlier test (GraphPad QuickCalcs, GraphPad Software, San Diego, California, USA).

Results

Data received
Results were received from 7 laboratories giving a total of 9 data sets (comprising 37 assays), for the Total FXIII-B subunit antigen potency assessment. All laboratories used the recommended ELISA for which specific monoclonal antibodies were provided. The data set also included 4 assays from a laboratory (6B) using an in-house ELISA method specific for Total FXIII-B subunit antigen and 5 assays from one laboratory which used frozen normal plasma pool (3A), in addition to using fresh normal plasma pools (3B).

Assay validity
Assays analysed at NIBSC were considered to be valid for linearity and parallelism, only if they satisfied the criteria set in section 5 (Statistical Analysis). In all cases, responses were log transformed in order to achieve the assumption of linearity. The following assays were excluded from the analysis: Laboratory 7 - assay 3, excluded due to non-parallelism and non-linearity of assay and assay 4, excluded due to excessive variability between replicates. A total of 35 valid assays were obtained. There was no evidence for consistent non-parallelism for the overall study.

Fresh and frozen local normal plasma pools (NP_1 & NP_2)
The Total FXIII-B subunit antigen potency results from each laboratory were expressed as units/ml relative to locally collected plasma pools NP.

Laboratories 1, 2, 3 (B), and 7 used fresh locally pooled plasma whereas the laboratories 3 (A), 4, 5 and 6 (A & B) used frozen locally pooled plasma. The difference between using fresh and frozen plasma was analysed for assays on the WHO 1st IS FXIII, Plasma (02/206) (TABLE 1). The frozen plasma gave a slightly higher Total FXIII-B subunit antigen potency [Geometric Mean=1.00 units/ml; n=21 assays; 95% confidence interval: 0.91 - 1.09; GCV%= 21.8] compared to freshly collected plasma [Geometric Mean=0.95 units/ml; n=14 assays; 95% confidence interval: 0.89 - 1.02; GCV%= 12.3], by ~ 5%. However, statistical analysis showed that there was no significant difference between the means obtained when using fresh or frozen plasma (p=0.36) and therefore these laboratories were not separated or excluded from overall estimates and analysis.

Total factor XIII-B subunit antigen potency estimates for the WHO 1st IS FXIII, Plasma (02/206) relative to locally collected plasma pools
The laboratory geometric mean Total Factor XIII-B subunit antigen potency estimates, the intra- and inter-laboratory %GCVs, relative to locally collected plasma pools are shown in TABLE 2.

Individual laboratory mean estimates are shown in graphical form in Figure 1. Each point on the graph represents the geometric mean estimate (from individual assays) from each laboratory as a relative potency compared to locally collected plasma pools.

The potency estimates calculated by each participating laboratory are shown in Appendix C.

Mean laboratory estimates for Total FXIII-B subunit antigen ranged from 0.83 to 1.29 units/ml with an overall geometric mean of 0.98 units/ml (n=9) and 95% confidence limits of 0.86 to 1.11 units/ml (TABLE 2 & Figure 1). Intra-laboratory variability (within-laboratory GCV%) ranged from 2.4% to 10.4% with majority of the laboratories having GCV values < 8%. Overall inter-laboratory variability (between-laboratory GCV%) was 18.3%. There were no outlying results

Discussion

In accordance with the precedent set for other International Standards, the Total FXIII-B subunit antigen calibration of the WHO 1st International Standard FXIII, plasma was carried out by assays against locally collected normal plasma pools, which were assigned an arbitrary value of 1.0 unit of Total FXIII-B subunit antigen per ml. A total number of 142 donors were used by the participating laboratories for the preparation of the normal plasma pools and it is assumed that this large number should cause the Total FXIII-B subunit unit to approximate to the population average.

Estimates of Total FXIII-B subunit antigen, relative to locally collected normal pooled plasmas, gave an overall mean value of 0.98 units/ml, which was very similar to the assigned FXIII A2B2 antigen potency of 0.93 IU/ml[1]. A reasonably good agreement between laboratories was also observed, despite each laboratory using different plasma pools, with an inter-laboratory variability (GCV%) of 18.3%. which compares well to the GCV% value of 16.4% obtained for the FXIII A2B2 antigen value assignment study[1].

Proposal for potency assignment
It is proposed that the WHO 1st IS Factor XIII plasma (NIBSC code 02/206) be assigned a Total FXIII-B subunit antigen value of 0.98 IU/ampoule.

Stability of WHO 1st IS FXIII Plasma (02/206) for the FXIII-B subunit antigen analyte

Real-time stability study - 17 years storage
Long-term storage allowed us to carry out a real-time stability study, at NIBSC, to asess the stability of the the FXIII-B subunit antigen analyte in the WHO 1st IS FXIII Plasma (02/206). This entailed testing of ampoules of the WHO 1st IS FXIII Plasma (02/206) stored at -20°C (bulk storage temperature), by assays relative to ampoules stored at -70°C (where an arbitrary value 1.00 is assigned to the -70°C ampoules). Ampoules of the WHO 1st IS FXIII Plasma (02/206) were placed into storage at -20°C and -70°C for the real-time stability study in 2002 and withdrawn after 17 years storage for FXIII-B subunit antigen assays. Three independent potency estimates were obtained using the FXIII-B subunit antigen ELISA described above.

The mean residual potencies for ampoules stored at -20°C (bulk storage temperature), expressed relative to ampoules stored at -70°C using an arbitrary value of 1.00, are given in TABLE 3. No loss in potency of the FXIII-B subunit antigen analyte was observed in the -20°C stored samples compared to samples stored at -70°C. This represents extremely good stability of the FXIII-B subunit antigen analyte in the WHO 1st IS FXIII Plasma (02/206) when stored at -20°C (bulk storage temperature), particularly as the standard was held in bulk storage for 17 years.

Bench stability following reconstitution
Although the Instructions for Use (IFU, see Appendix D) 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 International Standards for blood coagulation factors it is recommended that the standard is transferred, after reconstitution, to a plastic tube. Recommendations for the storage after reconstitution have been limited to the period of storage on melting ice since local ambient temperature can vary considerably.

The mean FXIII-B subunit antigen potency results from three separate independent tests performed at NIBSC, indicated that 92%, 90%, 95% and 97% of the starting potency of FXIII-B subunit antigen analyte was retained for the freshly reconstituted proposed standard when stored on melting ice in plastic tubes, for 0.5, 1, 2, and 4 hours, respectively. Based on this, a conservative estimate of a 3 hour maximum storage period is recommended, which is sufficient for numerous assays to be performed. The use of frozen aliquots of the WHO 1st IS FXIII Plasma (02/206) for the estimation of FXIII-B subunit antigen potency is not recommended.

Conclusion
The above real-time and bench stability studies indicate good stability and preservation of the FXIII-B subunit antigen analyte in the WHO 1st IS FXIII Plasma (02/206).

Comments from participants and the ISTH/SSC Factor XIII and Fibrinogen Subcommittee

Following a response questionnaire that was sent out to the study participants, responses were received from all laboratories (except one), all of whom approved the proposal that the WHO 1st IS Factor XIII plasma (NIBSC code 02/206) be assigned a Total FXIII-B subunit antigen value of 0.98 IU/ampoule.

There were additional comments from one participant:

Comment 1: Maybe I forgot to mention that we also used frozen pools. Please consider this for the evaluation.

NIBSC Response*:* We have re-analysed the comparison of the study data using fresh vs frozen plasma pools, with your laboratory as using frozen plasma pools and still there was no significant difference between the two. We have amended the report accordingly:-

"The frozen plasma gave a slightly higher Total FXIII-B subunit antigen potency [Geometric Mean=1.00 units/ml; n=21 assays ; 95% confidence interval: 0.91 -1.09; GCV%= 21.8] compared to freshly collected plasma [Geometric Mean=0.95 units/ml; n=14 assays; 95% confidence interval: 0.89 - 1.02; GCV%= 12.3], by ~ 5%. However, statistical analysis showed that there was no significant difference between the means obtained when using fresh or frozen plasma (p=0.36) and therefore these laboratories were not separated or excluded from overall estimates and analysis."

Comment 2: We have the impression that the contribution of laboratory 6 with two values 1.22 and 1.29 may have too much weight. Should we consider only results with the provided ELISA?

NIBSC Response: We have considered the contribution of laboratory 6 with two values but have decided to include both sets of data for the following reasons:

a. There are more than one laboratory contributing 2 sets of data and therefore we could not exclude a single laboratory's data. Furthermore, data from the in-house method (6B) was in fact found to be in a closer agreement to the Overall Mean compared to data from using the provided ELISA (6A).
b. The data obtained by laboratory 6 were not found to be outliers.
c. WHO standards are normally designed for calibration and standardisation using as many valid methods as possible and as such, using another second method, validates the study and gives a more robust data for value assignment.

Comment 3: The results from Laboratory 3 show that there are in principle small differences between frozen and fresh pools. This is in agreement with your statistical considerations. Therefore, we agree to combine the results.

NIBSC Response: Thank you for agreeing with the combination of the results. Comparison of data from all the laboratories in fact show that there is no statistical significant difference (p=0.36) between using fresh vs frozen plasma pools.

Comment 4: In the legend of the table two it is stated that the intra-lab variability is given. The table, however, gives the intra-assay GCV. Correct?

NIBSC Response: Thank you for pointing this out. This was a typographical error and we have amended the text in TABLE 2, accordingly.

In addition, the report was sent to 13 SSC Experts (via the WHO-ISTH Liaison Group), for further review. Responses were received by 6 SSC Experts all of whom agreed with the proposal. There were also additional comments from the SSC Experts to which the following responses were made and was accepted by the respective Experts:

Expert 1
Comment 1: A limitation of the study is the fact that it is mainly driven by one method.

***NIBSC Response**:* Although majority of the data is from one method, data was also included for a second ELISA method (Lab 6B), currently not available commercially. It is important to emphasise that at the time when the study was carried out there were no other specific antibodies/method available to measure Total FXIII-B subunit (both Bound and Free FXIII-B subunits) in plasma. In fact, following discussion at the SSC FXIII/Fibrinogen Subcommittee in 2016, requests were made to develop monoclonal antibodies specifically and this was achieved by Drs Muszbek and Katona, who then kindly provided these antibodies for the study. These antibodies were assessed and validated in a Pilot/Feasibility study involving 3 laboratories and the method made available for the current WHO calibration study.

Comment 2: Due to the relative high inter-lab variation there is a large confidence interval around the assigned value. This will affect to a similar extend the calculations of the measurement uncertainty by individual laboratories.

Comment 3: I would suggest that the SSC adopt the concept of uncertainty in the value assignment of their standards.

***NIBSC Response**:* WHO International Standards (ISs) are primary reference standards and are of the highest order standards against which other secondary standards are calibrated. As such uncertainty values are not assigned to WHO ISs. The reason for this is that, in a strict metrological sense, the replacement Unit is defined by the contents of the ampoule of the new standard. Every effort is made to maintain the continuity of the Unit, but the replacement Unit is not formally traceable to the previous ISs, only to the physical content of the replacement IS. For this reason, no uncertainty of measurement is applied to the IS.

Expert 2
Comment 1: an affiliation for the senior author is missing

***NIBSC Response**:* Affiliations of all authors were cited on page 1 and in Appendix A (page 12), we could not see any missing. If the comment is relating to Dr Peter Rigsby, his affiliation (no. 5) was indeed cited but for clarification purposes this has been rationalized on page 1 (reduced to 3 affiliations).

Comment 2: It is not fully clear to me why freshly collected plasma pools were used. It is stated (p3) that 142 donors were used to prepare the local pools, with a reference to table 1. However, table 1 does not contain information in the number of donors used for the fresh and frozen pools. I would suggest to add the number of donors used for each assay in table 2. Although the protocol (p14) suggests a minimum of 8 donors (unclear what this number is based on – a much higher number is thought to be required to set reference values), it is unclear whether this minimal amount was also achieved by the labs using fresh pools. Also the number of participants in the frozen pools is of interest.

***NIBSC Response**:* Although a previous feasibility/pilot study carried out to validate the ELISA method showed no difference between using fresh vs frozen plasma pools, it was decided to confirm this finding with the larger number of participants / larger number of plasma pools, in this study. We have however taken the comments on board and have included additional

information in Table 1 & Table 2 as requested, with number of donors used for the fresh & frozen pools and type and number pools for each data set, respectively. A minimum of 8 donors suggested for fresh plasma pools was based on previous WHO 1st IS FXIII study (see attached), where this number was deemed practicable but also suitable for such a small study. Furthermore, the minimum amount of 8 donors was indeed achieved by laboratories using fresh pools, as clarified in Table 2. The number of participants in the frozen pools are also clarified in Tables 1 & 2.

Expert 3
***Comment* 1**: A well-executed study that provides the first standard for FXIII-B. This will be useful for characterization of FXIII deficiency.

NIBSC Response*:* Thank you for your comments.

Expert 4
Comment 1: I have reservations about this material/study. It differs in several important ways form the usual approach. Only a single method was used. It may or may not be an appropriate potency for sure with other methods in future.

NIBSC Response*:* Although majority of the data is from one method, data was also included for a second ELISA method (Lab 6B), currently not available commercially. It is important to emphasise that at the time when the study was carried out there were no other specific antibodies/method available to measure Total FXIII-B subunit (both Bound and Free FXIII-B subunits) in plasma. In fact, following discussion at the SSC FXIII/Fibrinogen Subcommittee in 2016, requests were made to develop monoclonal antibodies specifically and this was achieved by Drs Muszbek and Katona, who then kindly provided these antibodies for the study. These antibodies were assessed and validated in a Pilot/Feasibility study involving 3 laboratories and the method made available for the current WHO calibration study.
As, for comments related to other methods in the future, it is even more important to have a reference standard/unit calibrated before development of new methods in order to calibrate/align the new method with correct unitage with minimal interlaboratory variability.

Comment 2: the material/method seems to be provided by a company (includes the word Ltd). This risks using the SSC/WHO process to give financial advantage. When companies donated materials for thromboplastin standards the donor was not identified to avoid this.

NIBSC Response*:* It is not our intention to give financial advantage to any company who donate material to help develop WHO International Standards, however they do need to be acknowledged for their donation. Nevertheless, we have taken aboard this comment and for clarification purposes, we have removed the commercial name (including the word "Ltd") from the main texts in the report but have retained it in the acknowledgement section, following precedence set for previous WHO IS establishment, including the thromboplastin standard mentioned where the donor was indeed identified (on page 5 and 10 of the WHO/ECBS Report WHO/BS/2016.2294).

Comment 3: There is no common sample which is puzzling since all similar studies I am aware of have done this to assess between lab variability. SSC standard lot 4 would have been an obvious candidate

NIBSC Response: This comment is a valid one. We had indeed considered a common plasma sample such as Lot#4/Lot#5, however due to the limitations of the assay reagents available and the logistics of the assay design (see Plate Layout in Appendix B, page 22), it was decided that information on local internal control included in the study would be more valuable and beneficial for future such studies than to include a common plasma. This study design was presented, discussed and reviewed at the ISTH/SSC FXIII/Fibrinogen Subcommittee meeting previously (Dublin 2018) and this approach was endorsed with no objections at the time. However, we do acknowledge that a common sample would help in assessing between lab variability.

Comment 4: I was not sure about the fresh versus frozen normal comparison – numbers in each group ?

NIBSC Response: Although a previous feasibility/pilot study carried out to validate the ELISA method showed no difference between using fresh vs frozen plasma pools, it was decided to confirm this finding with the larger number of participants / larger number of plasma pools, in this study. We have however taken the comments on board and have included additional information in TABLE 1 & TABLE 2 as requested, with number of donors used for the fresh & frozen pools and type and number pools for each data set, respectively.

Comment 5: Typo in table on page 3 about potency – 9.3 /9.1 IU/ml – and 0.91/0.93 IU/ml lower down.

NIBSC Response: Thank you for pointing this out. We have corrected this typographical error accordingly in on page 3 to 0.91/0.93 IU/ml for Activity potency and A2B2 antigen potency, respectively. This was for information only and these values were not used in the study in any way.

Review by the Scientific and Standardization Committee (SSC) of the International Society on Thrombosis and Haemostasis

The above study together with responses to all the comments received were reviewed and the project endorsed at the SSC Board meeting (the 65th SSC Meeting in Melbourne, 8th July 2019)

Proposal and recommendation to the ECBS

It is proposed that the current WHO 1st International Standard (IS) Factor XIII (FXIII) Plasma (02/206) be additionally assigned with a Total FXIII-B subunit antigen potency of 0.98 IU/ml.

References

1. Raut S, Merton RE, Rigsby P, Muszbek L, Seitz R, Arie¨ ns RAS, Barrowcliffe TW, Ichinose A, on behalf of the ISTH/SSC Factor XIII Subcommittee and the Factor XIII Standardization Working Party. A collaborative study to establish the first International Standard for factor XIII plasma. J Thromb Haemost 2007; 5: 1923–9.
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Acknowledgements

The contributions of all the participants in the study are gratefully acknowledged. We are grateful to our colleagues in the Standards Division (Sara-Jane Holmes, James Ahearne, James Condron, Sharon Coughlan, Paul Bolton, Mark Harris, Michael Aziz, Kevin Griffin, Trevor Stickland, Jaimin Joshi) NIBSC, for help with the standards project and the dispatch of collaborative study samples to participants. We are very grateful to Labexpert Ltd. (Debrecen, Hungary) & Dr Katona & Professor Muszbek (University of Debrecen, Debrecen, Hungary) for their kind donation of ELISA materials (monoclonal antibodies to FXIII-B subunit) for the study.

TABLE 1. Geometric Mean Total FXIII-B subunit antigen potency estimates for WHO 1st IS FXIII Plasma (02/206) relative to fresh and frozen locally collected plasma pools, together with estimates of inter-laboratory variability (GCV%) and 95% confidence intervals.

Total FXIII-B subunit antigen potency estimates relative to FRESH locally collected Normal Plasma Pools [n = 80 no. of Donors]			**Total FXIII-B subunit antigen potency estimates relative to FROZEN locally collected Normal Plasma Pools [n = 62 no. of Donors]**		
No of Assays (n)	**Geometric Mean (units/ml) [95% Confidence Interval]**	**Inter-Lab GCV%**	**No of Assays (n)**	**Geometric Mean (units/ml) [95% Confidence Interval]**	**Inter-Lab GCV%**
14	**0.95 [0.89 - 1.02]**	**12.3**	**21**	**1.00 [0.91 - 1.09]**	**21.8**
Total no. of donors contributing to the Normal Plasma Pools collected = 142					

TABLE 2. Estimates of Total FXIII-B subunit antigen in the WHO 1st IS FXIII plasma (02/206) relative to locally collected Normal Plasma Pools (NP_1 & NP_2), together with estimates of intra-laboratory variability (GCV%) for individual laboratories, laboratory mean potency as % of Overall Mean, Overall Combined Geometric Mean (units/ml), inter-laboratory variability (GCV%) and 95% confidence interval.

Lab Code	Method	Potency Estimates vs N_1 (Day 1) [95% CL] (units/ml)	Potency Estimates vs N_2 (Day 2) [95% CL] (units/ml)	Type & Total No of Donors (n) for the Plasma Pools	No of Assays (n)	Combined Laboratory Geometric Mean (units/ml)	95% Confidence Limits	Potency as % of Overall Mean	Intra-Lab GCV(%)
1	Provided ELISA	1.12 [1.07-1.17] 1.15 [1.09-1.22]	1.11 [0.97-1.28] 0.99 [0.93-1.07]	Fresh n=16	4	1.09	0.99 - 1.21	112	6.8
2	Provided ELISA	0.86 [0.80-0.94] 0.85 [0.81-0.89]	0.89 [0.87-0.92] 0.89 [0.84-0.95]	Fresh n=20	4	0.87	0.84 - 0.91	90	2.4
3A	Provided ELISA	0.89 [0.80-0.99] 0.85 [0.70-1.03] 1.02 [0.95-1.09]	0.88 [0.76-1.02] 0.86 [0.64-1.15]	Frozen* n=28	5	0.90	0.82 - 0.98	92	7.6
3B	Provided ELISA	0.97 [0.74-1.27] 1.05 [0.98-1.13]	0.87 [0.74-1.04] 1.01 [0.98-1.13]	Fresh* n=28	4	0.97	0.86 - 1.10	99	8.3
4	Provided ELISA	0.84 [0.81-0.88] 0.91 [0.87-0.95]	0.84 [0.78-0.91] 0.87 [0.71-1.07]	Frozen n=22	4	0.87	0.82 - 0.92	89	3.6
5	Provided ELISA	0.77 [0.75-0.79] 0.89 [0.86-0.93]	0.80 [0.73-0.88] 0.88 [0.79-0.97]	Frozen n=23	4	0.83	0.74 - 0.93	85	7.3
6A	Provided ELISA	1.20 [1.00-1.44] 1.50 [1.43-1.57]	1.24 [1.18-1.31] 1.26 [1.07-1.49]	Frozen§ n=17	4	1.29	1.10 - 1.51	133	10.4
6B	In-House ELISA	1.18 [1.10-1.26] 1.32 [1.09-1.59]	1.18 [1.04-1.34] 1.22 [1.08-1.37]	Frozen§ n=17	4	1.22	1.13 - 1.33	125	5.3
7	Provided ELISA	0.83 [0.72-0.96] 0.85 [0.71-1.02]	- -	Fresh n=16	2	0.84	0.71 - 0.98	86	-
Overall Combined Geometric Mean = 0.98 units/ml (n=9) Inter-Lab GCV = 18.3% 95% Confidence Interval: [0.86 - 1.11]									

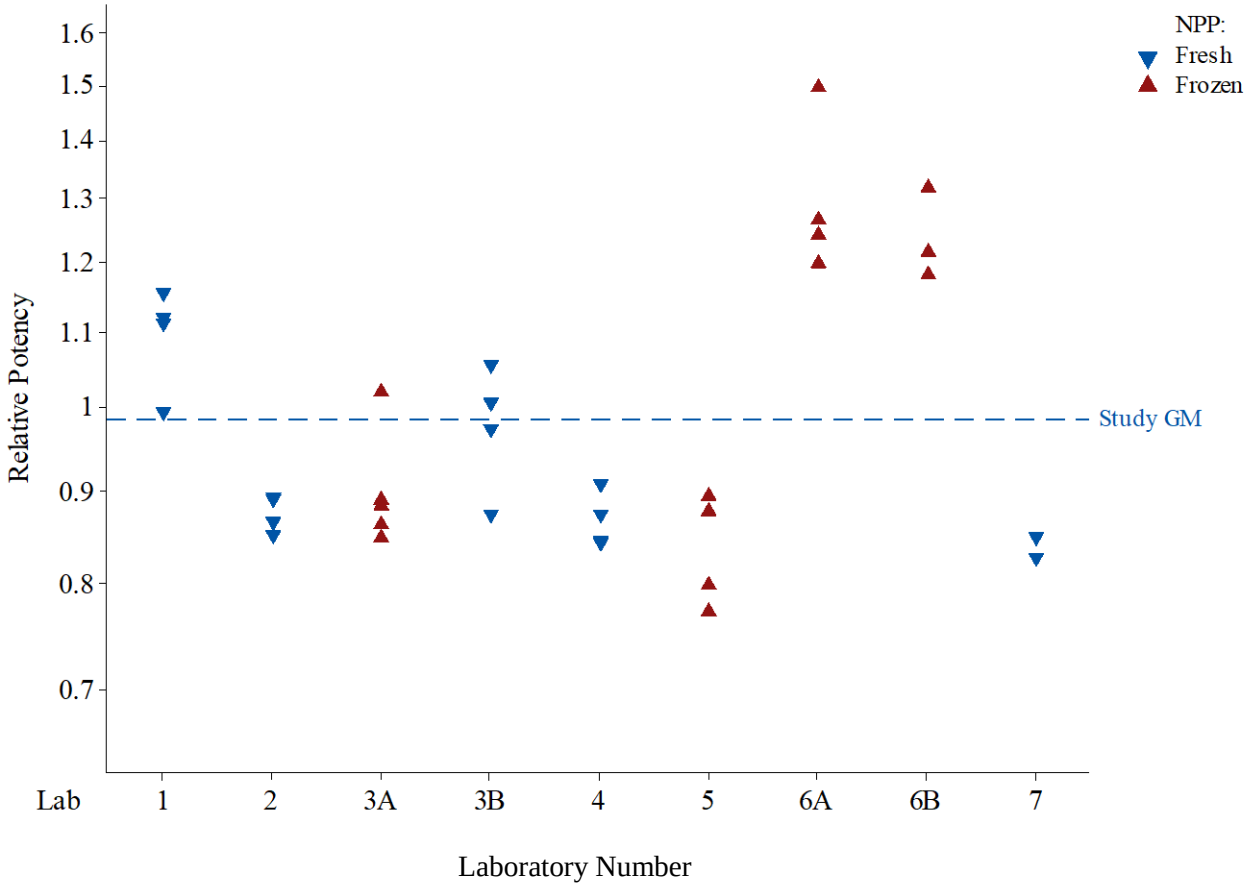
*Same donors for both Fresh and Frozen plasma pools; § Same donors for Frozen plasma pools

TABLE 3. FXIII-B subunit antigen potency estimates in ampoules of WHO 1st IS FXIII Plasma (02/206) stored for 17 years at -20°C relative to ampoules stored at -70°C (assigned 1.00).

Ampoule/Assay Number	**Residual FXIII-B subunit antigen potencies of -20°C ampoules after storage for 17 years (relative to -70°C ampoules) [95% Confidence Limits]**
1	0.964 [0.873 - 1.064]
2	1.167 [1.014 - 1.348]
3	0.909 [0.796 - 1.037]
Geometric Mean† [95% Confidence Limits]	**1.001 [0.896 - 1.119]**

† Based on semi-weighted combination of potency estimates

Figure 1. Graph showing individual laboratory's mean Total FXIII-B subunit antigen potency estimates for the WHO 1st IS FXIII Plasma (02/206) relative to locally collected plasma pools (Fresh ▼ ; Frozen ▲). Each point represents laboratory's individual assay geometric mean, expressed as units/ml. Dashed line represents the study Overall Combined Geometric Mean.



Appendix A

Participating Laboratories

Robert Ariens & Helen McPherson, University of Leeds, Leeds, UK

Johannes Dodt, Department of Haematology and Transfusion Medicine, Paul-Ehrlich-Institut, Federal Institute for Vaccines and Biomedicines, Langen, GERMANY

Akitada Ichinose, Department of Health Policy Science & Public Health, Yamagata University School of Medicine, Yamagata, JAPAN

Caroline Lawrence & Vivienne Gibson, Dept of Haematology, Glasgow Royal Infirmary, Glasgow, SCOTLAND

László Muszbek, Eva Katona, University of Debrecen, Medical Faculty, Division of Clinical Laboratory Science, Department of Laboratory Medicine, Debrecen, HUNGARY

Verena Schroeder, Experimental Haemostasis Group, Department for BioMedical Research, University of Bern, Bern, SWITZERLAND

Sanj Raut, Carmen Coxon, Andrew Riches-Duit, Peter Rigsby, NIBSC, Hertfordshire, UK

Appendix B

A Collaborative Study to Additionally Assign Value for Total Factor XIII-B Subunit to the WHO 1st IS for FXIII Plasma (02/206)

Study Protocol

Aim of Study

To calibrate of the WHO 1st IS for FXIII plasma (02/206) for total FXIII-B subunit, using a TOTAL FXIII-B ELISA, relative to locally collected plasma pools

Materials

The following test material is provided (4 test ampoules plus 1 spare):

> **WHO 1st IS FXIII Plasma (1st IS) [NIBSC code: 02/206], activity potency of 0.91 IU/ampoule; antigen (A2B2) potency of 0.93 IU/ampoule.**

In addition, Total FXIII-B Subunit Antigen assay kit antibodies (kindly provided by Dr Katona & Professor Muszbek, Labexpert Ltd, Debrecen, Hungary) are included with the samples:

- 2 vials of MAb1 (Biotinylated Anti-Total FXIII-B) and
- 2 vials of MAb3 (HRP-Labelled Anti-Total FXIII-B).

Locally Provided:
Each laboratory will be required to prepare separate **local plasma pools (NP_1 & NP_2)** for the study as instructed below.

Furthermore, each lab will need to purchase/use their own 96 well streptavidin coated microtiter plates and chemicals for the assay as outlined in the Kit Instructions (Appendix I) provided.

Optional:
Each laboratory, should they wish to do so, can also include their own **Internal Control (IC)** FXIII plasma sample or a secondary in-house sample in the study, in order to determine limits of assay acceptability/assay validity criteria, for their own use in future [e.g. main definitive collaborative study to value assign for Total FXIII B subunit to the WHO 1st IS FXIII Plasma (02/206)].

Reconstitution and Storage of WHO 1st IS FXIII Plasma (02/206)

i) On arrival of shipment, store the ampouled WHO 1st IS material (**1st IS**) at -20°C.
ii) Reconstitute at room temperature with 1.0 ml of distilled H_2O; see instructions (IFU) enclosed for the WHO 1st IS FXIII plasma sample.
iii) Mix gently and thoroughly to dissolve and transfer entire contents of ampoule to a plastic tube.
iv) Keep samples at room temperature during assays.

Storage of Kit Antibodies (MAb1 & MAb3)

i) On arrival of shipment, store the MAb vials at +4ºC.
ii) Use as directed in Kit Instructions enclosed (Appendix I).
iii) Keep antibodies at +4ºC for the duration of each set of assays, unless stated in the Kit Instructions.

Dilution of Samples (*Assay dilutions):*

All working dilutions of each sample should be carried out using routine buffers containing 0.5% (5 mg/ml) clinical grade human albumin (or if not available, bovine serum albumin of the highest purity possible). At least three dilutions should be made (see Kit Instructions - Appendix I & section on assay design). After making all assay dilutions, discard reconstituted material and make up a fresh ampoule for the next assay.

Collection of fresh normal plasma (for local plasma pools NP_1 & NP_2)

Collect fresh normal plasma on two separate days to prepare pools **NP_1** and **NP_2**. The method of collection for the fresh normal plasma is an important part of the study and should be standardised as far as possible according to the following protocol.

Donors: Normal healthy volunteers. Take blood from as many different individuals as possible, on two separate days. If possible, use a minimum of 8 different donors for each pool; if this is not possible, some of the same individuals can be used again, but the aim is to have as many different donors as possible.

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

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

Storage: Keep plasma pool in a plastic stoppered tube at +4ºC for the duration of each set of assays.

Assay Methods

For the total FXIII-B subunit antigen (Ag) assays, laboratories are asked to use the provided kit antibodies (Labexpert Ltd). Please follow Kit Instructions (Appendix I) enclosed.

Number of Assays and Ampoules

Four independent assays are requested for the ELISA method from each laboratory, to be carried over 2 separate days (Normal Plasma Pool **NP_1** – Day 1; Normal Plasma Pool **NP_2** - Day 2), rather than all on the same day. Only one set of 5 ampoules (4 test ampoules, 1 spare) of the test material is provided. A separate ampoule of the test sample material should be used for each potency (Total FXIII-B Subunit Antigen ELISA) assay.

Assay Design

For each sample (1^{st}IS, NP, IC) make a fresh set of 3 or more doubling working dilutions (e.g. 1/1, 1/2, 1/4, 1/8). Where a sample is repeated twice within an assay in the assay design below, a replicate (duplicate) fresh set of dilutions from the same ampoule/original sample should be made. Remember to use a fresh set of test 1^{st} IS ampoule/Normal Plasma Pool/IC sample for each assay. Please perform assays using the following assay design only, as detailed in the exemplar Plate Layout in Appendix II.

6 Samples per Assay

A balance order of testing should be adopted wherever possible:

Plasma Pool	Assay No (Amp No)	Order of Testing					
Day 1 NP_1	**1**	1^{st}IS	NP_1	IC	IC	NP_1	1^{st} IS
	2	IC	1^{st}IS	NP_1	NP_1	1^{st}IS	IC
Day 2 NP_2	**3**	NP_2	IC	1^{st}IS	1^{st}IS	IC	NP_2
	4	1^{st}IS	NP_2	IC	IC	NP_2	1^{st} IS

Please refer to exemplar Plate Layout in Appendix II.

Analysis & Submission of Results

Please return all raw data (i.e. ΔOD or OD values) including all details of dilutions performed, using the Excel Results sheets provided (sent separately via e-mail). In addition, it would be helpful if each laboratory can calculate their own Total FXIII-B subunit potencies against the locally collected plasma pools, assuming the latter to have a Total FXIII-B Subunit antigen potency of 1 u/ml (see Excel Sheet). Assays should be analysed using parallel line or slope ratio analysis.

Please submit all the results, to **Dr S Raut, Haemostasis Section, Biotherapeutics Group, National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Potters Bar, Hertfordshire, EN6 3QG, UK.**
e-mail: sanj.raut@nibsc.org

Appendix I

Instructions: TOTAL FXIII-B ELISA

ELISA COMPONENTS

1. **Capture antibody (MAb1) -** Biotinylated anti-FXIII-B mouse monoclonal antibody, lyophilized. Store at 2-8 °C.

2. **Detection antibody (MAb3) -** HRP-conjugated anti-FXIII-B mouse monoclonal antibody, 1 mL in stabilizing solution. Store at 2-8 °C.

Capture and detection antibodies are directed against different epitopes on FXIII-B.

3. **Washing buffer** - 0.14 mol/L NaCl, 3 mmol/L KH_2PO_4, 12 mmol/L Na_2HPO_4, 0.05% Tween 20, pH: 7.2. Store at 2-8 °C.

4. **Assay diluent** - 0.5 mol/L NaCl, 3 mmol/L KH_2PO_4, 12 mmol/L Na_2HPO_4, 0.05% Tween 20, 0.5% BSA, pH: 7.2. Store at -20 °C.

5. **Substrate solution** (*Not provided*) - Tetramethylbenzidine (TMB) substrate, ready for use (One Component HRP Microwell Substrate, DIARECT, Freiburg, Germany). Store at 2-8 °C.

6. **Stop solution** - 2 mol/L H_2SO_4.

7. **ELISA plates** (*Not provided*) - 12 strips with 8 wells each, coated with streptavidin, ready to use (KaiSA96 Lockweel plate, Kaivogen, Turku, Finland). After warming to room temperature (25-30 minutes) open the aluminium pouch to remove the strips, just before use. Strips are breakable. If only part of the strips/wells are used, store the remaining strips/wells at 2-8 °C in the original package with desiccator.

8. **Adhesive film for ELISA plates -** (*Not provided*).

Capture antibody (MAb1) working solution:

After warming to room temperature reconstitute lyophilized antibody with 100 µL of distilled water and allow to equilibrate for 20 min at room temperature. Then add 400 µL assay diluent, mix thoroughly, without vortexing (this is now referred to as reconstituted antibody). One vial is sufficient for one plate; if only part of the plate is used the remaining reconstituted antibody solution is stable for 4 weeks when stored at 2-8 °C. Freezing is not recommended! Just before use, the reconstituted capture antibody solution must be further diluted (1:20) with assay diluent. (For 48 wells: add 200 µL of reconstituted capture antibody to 3800 µL of assay diluent).

Detection antibody (MAb3) working solution:

A 1:10 dilution of the detection antibody should be prepared with assay diluent immediately prior to use. (For 48 wells: add 400 µL of detection antibody solution to 3600 µL of assay diluent)

Dilution of samples

Patients' and quality control plasmas are normally tested at a dilution of 1:1000.

Initial Dilutions

The following two-step (**1:250**) dilution scheme is recommended for the initial dilutions of test samples (**NP**, **1st IS** & **IC** plasma):

1. 20 µL plasma + 480 µL assay diluent = 1:25 plasma dilution
2. 50 µl of 1:25 pre-diluted plasma + 450 µl assay diluent = 1:10 dilution

Initial plasma dilution is therefore 1:250.

Working Dilutions

Using the initial dilution of test samples (above), the following working dilutions are recommended:

1. 1/1 dilution: 200 µL of "neat" 1:250 initial dilution (from above)
   [= 1:250 final plasma dilution].

2. 1/2 dilution: 100 µL of "neat" 1:250 plasma dilution + 100 µL assay diluent
   [= 1:500 final plasma dilution]

3. 1/4 dilution: 100 µL of 1:500 plasma dilution + 100 µL assay diluent
   [= 1:1000 final plasma dilution]

4. 1/8 dilution: 100 µL of 1:1000 plasma dilution + 100 µL assay diluent
   [= 1:2000 final plasma dilution]

ASSAY PROCEDURE

1. A pipetting scheme is outlined in Plate Layout in Appendix II. This scheme is designed to ensure that each sample is assayed in duplicate and is the preferred scheme for this assay. If using strips, remove the required number of strips as calculated from the pipetting scheme, and replace the remaining strips into the pouch with the desiccant.
2. Wash the wells twice with 300 µL washing buffer (pre-wash) (**Note** 1).
3. To the appropriate well, pipette 70 µL of the diluted test samples (**NP**, **1st IS** & **IC** plasma) and add 70 µL of diluted detection antibody (MAb3), and 70 µL of diluted capture antibody (MAb1) are added to each well (total volume = 210 µl)
4. Seal the plates and incubate for 1 hour at room temperature with constant, gentle agitation.
5. Wash the wells 4 times with 300 µL washing buffer.
6. After removing the wash buffer, add 200 µL substrate solution to each well (**Notes** 2 and 3).
7. Re-seal the plate and further incubate for 30 minutes at room temperature

8. Stop the reaction by adding 50 µL 2 mol/L H_2SO_4 (**Note** 3).
9. Read absorbance at 450 nm in a microplate reader within 1 hour.

ELISA Summary Table:

ELISA Steps	**Procedure**	**Volume used / Incubation time**
PRE-WASHING of test strips	Wash with washing buffer	2x300 µL
INCUBATION of diluted sample with detection **and** capture antibody	Pipette diluted samples into test wells	70 µL
	Add diluted detection antibody	70 µL
	Add diluted capture antibody	70 µL
	Seal the plate and incubate at room temperature with constant gentle shaking!	60 minutes
WASHING	Wash with washing buffer	4x300 µL
INCUBATION with substrate	TMB substrate (ready to use)	200 µL
	Incubate at room temperature	30 minutes
STOPPING the reaction	Pipette stop solution into the well	50 µL
MEASUREMENT	ELISA-Reader, 450 nm	within1 hour

Notes:

1. Removing excess streptavidin loosely bound to the plate by pre-washing improves assay precision. For wash steps, ensure that each well is filled, and then completely emptied (tap the inverted plate several times on absorbent paper). Do not let the wells to dry out and avoid strong direct light.

2. The TMB substrate is very sensitive to contamination; do not pipette TMB solution directly from the bottle but transfer the required quantity into a separate container and use this portion of the solution for the assay. Do not pour the remaining TMB solution back into the original bottle!

3. TMB and 2 mol/L sulphuric acid should be handled with care. If skin becomes contaminated, they are to be removed by repeated rinsing with water.

Appendix II

A Collaborative Study to Additionally Assign Value for Total Factor XIII-B subunit to the WHO 1st IS for FXIII Plasma (02/206)

48 WELL PLATE LAYOUTS:

Day 1, NP_1, ELISA 1

	1	2	3	4	5	6
A	**1stIS 1/1** dilution 1	**1stIS 1/1** dilution 1	**IC 1/1** dilution 1	**IC 1/1** dilution 1	**NP_1 1/1** dilution 2	**NP_1 1/1** dilution 2
B	**1stIS 1/2** dilution 1	**1stIS 1/2** dilution 1	**IC 1/2** dilution 1	**IC 1/2** dilution 1	**NP_1 1/2** dilution 2	**NP_1 1/2** dilution 2
C	**1stIS 1/4** dilution 1	**1stIS 1/4** dilution 1	**IC 1/4** dilution 1	**IC 1/4** dilution 1	**NP_1 1/4** dilution 2	**NP_1 1/4** dilution 2
D	**1stIS 1/8** dilution 1	**1stIS 1/8** dilution 1	**IC 1/8** dilution 1	blank	**NP_1 1/8** dilution 2	**NP_1 1/8** dilution 2
E	**NP_1 1/1** dilution 1	**NP_1 1/1** dilution 1	**IC 1/1** dilution 2	**IC 1/1** dilution 2	**1stIS 1/1** dilution 2	**1stIS 1/1** dilution 2
F	**NP_1 1/2** dilution 1	**NP_1 1/2** dilution 1	**IC 1/2** dilution 2	**IC 1/2** dilution 2	**1stIS 1/2** dilution 2	**1stIS 1/2** dilution 2
G	**NP_1 1/4** dilution 1	**NP_1 1/4** dilution 1	**IC 1/4** dilution 2	**IC 1/4** dilution 2	**1stIS 1/4** dilution 2	**1stIS 1/4** dilution 2
H	**NP_1 1/8** dilution 1	**NP_1 1/8** dilution 1	**IC 1/8** dilution 2	blank	**1stIS 1/8** dilution 2	**1stIS 1/8** dilution 2

Day 1, NP_1, ELISA 2

	1	2	3	4	5	6
A	**IC 1/1** dilution 1	**IC 1/1** dilution 1	**NP_1 1/1** dilution 1	**NP_1 1/1** dilution 1	**1stIS 1/1** dilution 2	**1stIS 1/1** dilution 2
B	**IC 1/2** dilution 1	**IC 1/2** dilution 1	**NP_1 1/2** dilution 1	**NP_1 1/2** dilution 1	**1stIS 1/2** dilution 2	**1stIS 1/2** dilution 2
C	**IC 1/4** dilution 1	**IC 1/4** dilution 1	**NP_1 1/4** dilution 1	**NP_1 1/4** dilution 1	**1stIS 1/4** dilution 2	**1stIS 1/4** dilution 2
D	**IC 1/8** dilution 1	blank	**NP_1 1/8** dilution 1	**NP_1 1/8** dilution 1	**1stIS 1/8** dilution 2	**1stIS 1/8** dilution 2
E	**1stIS 1/1** dilution 1	**1stIS 1/1** dilution 1	**NP_1 1/1** dilution 2	**NP_1 1/1** dilution 2	**IC 1/1** dilution 2	**IC 1/1** dilution 2
F	**1stIS 1/2** dilution 1	**1stIS 1/2** dilution 1	**NP_1 1/2** dilution 2	**NP_1 1/2** dilution 2	**IC 1/2** dilution 2	**IC 1/2** dilution 2
G	**1stIS 1/4** dilution 1	**1stIS 1/4** dilution 1	**NP_1 1/4** dilution 2	**NP_1 1/4** dilution 2	**IC 1/4** dilution 2	**IC 1/4** dilution 2
H	**1stIS 1/8** dilution 1	**1stIS 1/8** dilution 1	**NP_1 1/8** dilution 2	**NP_1 1/8** dilution 2	**IC 1/8** dilution 2	blank

Day 2, NP_2, ELISA 1

	1	2	3	4	5	6
A	**NP_2 1/1** dilution 1	**NP_2 1/1** dilution 1	**1st IS 1/1** dilution 1	**1st IS 1/1** dilution 1	**IC 1/1** dilution 2	**IC 1/1** dilution 2
B	**NP_2 1/2** dilution 1	**NP_2 1/2** dilution 1	**1st IS 1/2** dilution 1	**1st IS 1/2** dilution 1	**IC 1/2** dilution 2	**IC 1/2** dilution 2
C	**NP_2 1/4** dilution 1	**NP_2 1/4** dilution 1	**1st IS 1/4** dilution 1	**1st IS 1/4** dilution 1	**IC 1/4** dilution 2	**IC 1/4** dilution 2
D	**NP_2 1/8** dilution 1	**NP_2 1/8** dilution 1	**1st IS 1/8** dilution 1	**1st IS 1/8** dilution 1	**IC 1/8** dilution 2	blank
E	**IC 1/1** dilution 1	**IC 1/1** dilution 1	**1st IS 1/1** dilution 2	**1st IS 1/1** dilution 2	**NP_2 1/1** dilution 2	**NP_2 1/1** dilution 2
F	**IC 1/2** dilution 1	**IC 1/2** dilution 1	**1st IS 1/2** dilution 2	**1st IS 1/2** dilution 2	**NP_2 1/2** dilution 2	**NP_2 1/2** dilution 2
G	**IC 1/4** dilution 1	**IC 1/4** dilution 1	**1st IS 1/4** dilution 2	**1st IS 1/4** dilution 2	**NP_2 1/4** dilution 2	**NP_2 1/4** dilution 2
H	**IC 1/8** dilution 1	blank	**1st IS 1/8** dilution 2	**1st IS 1/8** dilution 2	**NP_2 1/8** dilution 2	**NP_2 1/8** dilution 2

Day 2, NP_2, ELISA 2

	1	2	3	4	5	6
A	**1st IS 1/1** dilution 1	**1st IS 1/1** dilution 1	**IC 1/1** dilution 1	**IC 1/1** dilution 1	**NP_2 1/1** dilution 2	**NP_2 1/1** dilution 2
B	**1st IS 1/2** dilution 1	**1st IS 1/2** dilution 1	**IC 1/2** dilution 1	**IC 1/2** dilution 1	**NP_2 1/2** dilution 2	**NP_2 1/2** dilution 2
C	**1st IS 1/4** dilution 1	**1st IS 1/4** dilution 1	**IC 1/4** dilution 1	**IC 1/4** dilution 1	**NP_2 1/4** dilution 2	**NP_2 1/4** dilution 2
D	**1st IS 1/8** dilution 1	**1st IS 1/8** dilution 1	**IC 1/8** dilution 1	blank	**NP_2 1/8** dilution 2	**NP_2 1/8** dilution 2
E	**NP_2 1/1** dilution 1	**NP_2 1/1** dilution 1	**IC 1/1** dilution 2	**IC 1/1** dilution 2	**1st IS 1/1** dilution 2	**1st IS 1/1** dilution 2
F	**NP_2 1/2** dilution 1	**NP_2 1/2** dilution 1	**IC 1/2** dilution 2	**IC 1/2** dilution 2	**1st IS 1/2** dilution 2	**1st IS 1/2** dilution 2
G	**NP_2 1/4** dilution 1	**NP_2 1/4** dilution 1	**IC 1/4** dilution 2	**IC 1/4** dilution 2	**1st IS 1/4** dilution 2	**1st IS 1/4** dilution 2
H	**NP_2 1/8** dilution 1	**NP_2 1/8** dilution 1	**IC 1/8** dilution 2	blank	**1st IS 1/8** dilution 2	**1st IS 1/8** dilution 2

Appendix C

Table A. Laboratories' own calculations for mean potency estimate for Total FXII-B subunit antigen relative to locally collected normal plasma pools.

Lab Code	Method	No of Assays (n)	Laboratory's Calculated Mean (units/ml)	95% Confidence Limits	Intra-Lab GCV(%)
1	Provided ELISA	4	-	-	-
2	Provided ELISA	4	0.89	0.86 - 0.92	2.1
3A	Provided ELISA	5	0.90	0.82 - 0.98	7.6
3B	Provided ELISA	4	0.97	0.86 - 1.10	8.3
4	Provided ELISA	4	0.86	0.82 - 0.90	2.2
5	Provided ELISA	4	0.80	0.71 - 0.90	7.8
6A	Provided ELISA	4	1.24	1.10 - 1.38	8.1
6B	In-House ELISA	4	1.14	1.06 - 1.22	4.0
7	Provided ELISA	4	0.76	0.60 - 1.07	26.2

Appendix D

Instructions For Use (IFU)

Medicines & Healthcare products Regulatory Agency

NIBSC Confidence in Biological Medicines

WHO International Standard
1st International Standard Factor XIII Plasma, Human
NIBSC code: 02/206
Instructions for use
(Version 7.00, Dated 04/03/2015)

1. INTENDED USE

The WHO 1st International Standard for Blood Coagulation Factor XIII (FXIII) Plasma consists of ampoules, coded 02/206, containing aliquots of a freeze-dried human plasma containing FXIII. This preparation was established by the Expert Committee on Biological Standardization (ECBS) of the World Health Organization in November 2004.

This standard is intended to be used in the measurement of FXIII, both activity and antigen (A2B2 complex & Total FXIII-B subunit), in plasma and is primarily intended for calibration of secondary and/or in-house working FXIII plasma standards.

The latest ECBS report is available from the WHO (www.who.int/biologicals). Document number: WHO/BS/2019.xxxx

2. CAUTION

This preparation is not for administration to humans or animals in the human food chain.

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.he plasma for this preparation was prepared from pools of human plasma in which every donation was tested and found negative for hepatitis B surface antigen, antibodies to HIV-1 and -2 and antibodies to hepatitis C. The filled candidate preparation was subsequently tested and found negative for HCV RNA by PCR.

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 biological activity and antigen content (A2B2 complex) of the 1st International Standard for Blood Coagulation FXIII Plasma (coded 02/206), was calibrated in INTERNATIONAL UNITS (IU), in an international collaborative study involving 23 laboratories in 10 countries. This standard was additionally calibrated for Total FXIII-B subunit antigen in a further international collaborative study involving 7 laboratories in 6 countries

The assigned potencies are:

FXIII activity potency - 0.91 IU per ampoule

FXIII A2B2 antigen potency - 0.93 IU per ampoule

Total FXIII-B subunit antigen potency - 0.98 IU per ampoule

4. CONTENTS

Country of origin of biological material: United Kingdom.

The 1st International Standard for Blood Coagulation FXIII, Plasma (coded 02/206), contains freeze-dried (1 mL) aliquots of a pooled human plasma containing Factor XIII.

Frozen units of plasma were thawed and pooled. 1 molar solution of HEPES was slowly added and the pool gently stirred to give a final concentration of 0.04M HEPES. The pooled buffered plasma was then distributed at 4°C into ampoules, coded 02/206 and the contents of the ampoules were freeze-dried under the conditions normally used for international biological standards[1].

5. STORAGE

Unopened ampoules should be stored at -20°C. After reconstitution, any unused material must be discarded, not frozen for later use.
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. The total contents of the ampoule should be reconstituted at room temperature with 1 ml distilled water, dissolved by gentle swirling to avoid froth and transferred immediately to a suitable plastic tube. The reconstituted Standard is stable for up to 2 hours at room temperature.

N.B. If using this Standard to calibrate Factor XIII concentrates, the test concentrates MUST be pre-diluted in FXIII deficient plasma, before making the assay dilutions. Assay dilution buffers should contain 1% albumin, preferably clinical grade.

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.

Accelerated degradation studies have shown that this standard is extremely stable both when stored at -20°C and at mailing temperatures. Predicted loss of both FXIII activity & FXIII antigen (A2B2 complex) when stored at -20°C were below 0.05% per year. Real-time stability studies, after 17 years storage at -20°C, has shown no loss in FXIII-B subunit antigen potency.

NIBSC follows the policy of WHO with respect to its reference materials.

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

World Health Organization

Medicines & Healthcare products Regulatory Agency

NIBSC Confidence in Biological Medicines

9. REFERENCES

1. Campbell P J. Procedures used for the production of biological standards and reference preparations. J Biol Standardization, 1974, 2, 259-267.

10. ACKNOWLEDGEMENTS

Are made to all the participants in the study and to the North London Blood Transfusion Centre for supplies of the candidate material for the study. We would also like to express our sincere thanks to ISTH/SSC FXIII Subcommittee and to the ISTH/SSC FXIII Standardisation Working Party (SWP) for their guidance.

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/standardisation/international_standards.aspx
Ordering standards from NIBSC:
http://www.nibsc.org/products/ordering.aspx
NIBSC Terms & Conditions:
http://www.nibsc.org/terms_and_conditions.aspx

12. CUSTOMER FEEDBACK

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

13. CITATION

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

14. MATERIAL SAFETY SHEET

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

hysical and Chemical properties		
Physical appearance: Freeze dried powder	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: 0.09g
Toxicity Statement: Non-toxic
Veterinary certificate or other statement if applicable. **Attached**: No

17. CERTIFICATE OF ANALYSIS

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

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