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AN INTERNATIONAL COLLABORATIVE STUDY TO ESTABLISH THE WHO 3RD INTERNATIONAL STANDARD FOR THROMBIN

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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 **10 August 2020** 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

Background: An international collaborative study was organised to calibrate a replacement for the WHO 2nd International Standard for Thrombin (01/580), stocks of which are running low. Twenty laboratories from 13 countries were asked to measure the potency of two candidate replacement standards relative to the 2nd IS (designated sample S), using their in-house plasma or fibrinogen clotting assays and/or chromogenic assays. Sample A (01/578) was used in the previous international collaborative study in 2002 to establish the 2nd IS and samples B and C were coded duplicates of a newly ampouled material donated by a manufacturer (coded 19/188).

Results: Nineteen laboratories contributed a total of 111 assays after exclusions, which comprised 91 clotting assays, 56 with fibrinogen and 35 with plasma. Of these, 52 were performed with automated coagulometers, 12 with manual coagulometers, and 27 with microtitre plate-based assays. Four laboratories performed chromogenic assays (16 assays in total) and one laboratory performed fluorogenic assays (4 assays). Variation between and within laboratories was low, with inter- and intra-laboratory geometric coefficient of variation (GCV) generally < 5 % for all assay methods and substrates. For sample A (01/578), potency estimates by clotting assays (101.1 IU/ampoule, inter-laboratory GCV = 3.5 %) were significantly lower than estimates by chromogenic assays (111.5 IU/ampoule, inter-laboratory GCV = 4.4 %), in good agreement with the 2002 study. The estimate by fluorogenic assay was midway between clotting and chromogenic.

Mean potency estimates for coded duplicate samples B and C (19/188) were almost identical and combined to give an overall mean potency of 90.4 IU/ampoule by clotting assay (inter-laboratory GCV = 3.9 %) and 88.1 IU/ampoule by chromogenic assay (inter-laboratory GCV = 2.5 %). The close ratio between clotting and chromogenic assay potency estimates for 19/188 suggests it has a higher α-thrombin content than 01/578 and is equivalent to the current IS (01/580). The single estimate by fluorogenic assay was in good agreement with clotting and chromogenic assays (89.9 IU/ampoule).

Accelerated degradation studies on 19/188 indicate the candidate international standard is very stable, in agreement with results for previous thrombin standards.

Conclusion: It is proposed that preparation 19/188 is a suitable replacement for the WHO 2nd IS (01/580) and is established as the 3rd IS for Thrombin, with a potency of 90 IU/ampoule, based on estimates from clotting assays.

INTRODUCTION

The first International Standard for thrombin endorsed by WHO was established in 1975 to define the unitage of thrombin in terms of biological activity (International Units, IU) (1). The standard was a partially purified preparation that also contained high amounts of proteolyzed forms of α-thrombin, known as β-and γ-thrombin. While α-thrombin clots fibrinogen, the clotting ability of β and γ forms is diminished and they are only active towards synthetic substrates. This necessitated the need for an α-thrombin standard, which was established in 1991 from a high-purity preparation (coded 89/588, the 1st IS for α-Thrombin) (2). In parallel, the United States National Institute of Health (NIH) had been developing thrombin standards defined by the "NIH" or US unit and given "Lot" designations. The NIH unit and IU were similar but not identical, with the ratio between the two units dependent on assay conditions (3). The widespread use of two standards with two units caused confusion among users of thrombin standards. In 2003 the US Thrombin Standard (Lot J) and WHO IS for α-Thrombin (89/588) were replaced by a single standard, calibrated in a single unit, and designated the WHO 2nd IS for Thrombin (01/580) and the US Standard Thrombin, Lot K (4).

The WHO 2nd IS for Thrombin (01/580) has been in high use since its establishment and is used by manufacturers of laboratory diagnostic and therapeutic thrombin products for potency assignment. These include the increasing use of fibrin sealant or "glue" kits as topical haemostats, sealants, or adhesives in surgical procedures and whose principal components are thrombin and fibrinogen (5).

Stocks of 01/580 are running low and a replacement is required. This report describes the international collaborative study to calibrate the WHO 3rd IS for Thrombin. Twenty laboratories took part in the study and measured the potency of two candidate materials (01/578 and 19/188) relative to the 2nd IS (01/580). It is proposed that 19/188 is the WHO 3rd International Standard for Thrombin with a potency of 90 IU/ampoule, based on estimates from clotting assays.

MATERIALS

One manufacturer kindly donated a preparation of α-thrombin (coded FEU045A). FEU045A was prepared by fractionation of pooled human plasma tested for virological markers and negative for HIV-1/HIV-2 antibody, HBs antigen, and HBV, HCV, and HIV-1/2 viral nucleic acid. The manufacturer reported FEU045A contained ~ 950 IU/mL thrombin (by fibrinogen clotting and synthetic chromogenic substrate) and approximately 22 mg/mL protein, the majority of which was human albumin, added as a stabiliser and negative for virological markers. FEU045A was shipped frozen to NIBSC and maintained at – 80 °C until reformulation. The bulk material (~ 950 mL) was added to 10 mM HEPES, pH 7.4, 150 mM NaCl, and human albumin to a final concentration of 5 mg/mL, giving a final thrombin concentration of approximately 90 IU/mL in a total of 10 L. In October 2019, 5 mL DIN ampoules were filled with 1 mL aliquots of the diluted material, lyophilised following NIBSC procedures, and coded 19/188. Ampoules are stored at NIBSC (Potters Bar, UK) at -20 °C.

Measurement of the mean oxygen head space after sealing served as a measure of ampoule integrity. This was measured non-invasively by frequency modulated spectroscopy (FMS

760, Lighthouse Instruments, Charlottesville, VA, USA), based upon the Near Infra-Red absorbance by oxygen at 760 nm when excited using a laser. Controls of 0% and 20% oxygen were tested before samples were analyzed to verify the method. Twelve ampoules were tested at random from each material; oxygen should be less than 1.14%.

Residual moisture content was measured for the same 12 ampoules per material using the coulometric Karl Fischer method in a dry box environment (Mitsubishi CA200, A1 Environsciences, Blyth, UK) with total moisture expressed as a percentage of the mean dry weight of the ampoule contents. Individual ampoules were opened in the dry box and reconstituted with approximately 1-3 ml Karl Fischer anolyte reagent which was then injected back into the Karl Fischer reaction cell and the water present in the sample determined coulometrically. Dry weight was determined for six ampoules per material weighed before and after drying, with the measured water expressed as a percentage of the dry weight.

A total of 9720 ampoules of 19/188 were available for use. Precision of the fill was monitored by check-weights evenly spaced throughout the total fill. The results are expressed as the % coefficient of variation (cv), where n is the number of ampoules sampled to determine each parameter: mean filling weight = 1.0070 g (cv = 0.14 %, n = 343); mean dry weight = 0.01643 g (cv = 1.16 %, n = 6); mean residual moisture content = 0.49 % (cv = 27.68 %, n = 12); mean oxygen headspace = 0.43 % (cv = 33.92 %, n = 12).

STUDY DESIGN

The study consisted of four samples, one of which was the WHO 2nd IS for Thrombin (designated sample S, 01/580), one of which was a candidate preparation used in the study to establish the 2nd IS (designated sample A, 01/578 in that study), and two anonymously coded duplicates of candidate preparation 19/188 (designated samples B and C). Participants were asked to perform 4 independent clotting (plasma/fibrinogen) or chromogenic assays to compare the potency of samples A, B, and C, relative to sample S, using fresh ampoules for each assay. In these assays, a four-point doubling dilution range of each thrombin sample is added to fibrinogen or plasma and clotting times recorded.

A results sheet was provided where participants were asked to record experimental details and clotting times or reaction rates. Participants were also asked to return raw data where possible, for example microtitre plate readouts in Excel formats in the form or absorbance versus time, so that complete analysis of all raw data could be performed at NIBSC.

METHODS

Laboratories were encouraged to use their own assay methods, following guidelines on dilution and randomisation regimes. In addition, example protocols were provided, which had been developed and validated at NIBSC as part of prior fitness-for-purpose testing of the candidate standards. These methods were suitable for use with plasma, fibrinogen, or chromogenic substrates, and are representative of methods in contemporary use. Formats included an automated coagulometer method on the ACLTop500/550 (Werfen, UK) and microtitre plate-based methods using plate readers. The study protocol and assay methods are included in Appendix 1.

For automated coagulometer methods, clotting times are defined by instrument-specific thresholds, usually a 10-15 % change in absorbance (at wavelengths between 405 to 660 nm) during the incubation period. For microtitre plate-based methods, it was initially requested that clotting times be reported as time to 50% clotting, or half maximal absorbance, similar to previous collaborative studies with thrombin-like snake venom proteases (6). However, for plasma clotting assays the time to 15 % clotting provides a better fit to the parallel line model, in line with automated coagulometer thresholds. For chromogenic substrates, rates of hydrolysis were calculated at NIBSC for absorbance changes (at 405 nm) up to 0.1 units, where raw data was available. Alternatively, rates were reported by the participants using their own analysis. To help with analysis of microtitre plate data, links were provided to online apps, to obtain clotting times or reaction rates (7). One laboratory also performed plate-based fluorogenic assays, in which the rate of hydrolysis of Z-Gly-Gly-Arg-AMC was measured using a fluorescent plate reader (excitation 390 nm and emission 460 nm).

Semi-automated or manual coagulometers were also included in the study. These methods measure physical properties of the clot rather than clot turbidity. For example, a steel ball is placed into a rotating cup containing the clotting sample. Fibrin clot formation impedes the movement of the ball, which is dislodged from the position of a magnetic sensor and recorded as the clotting time (performed by Lab 15). In a variation of this method, clotting time is defined as a threshold increase in viscosity, measured by monitoring the amplitude of an oscillating steel ball in a cuvette containing the clotting sample (performed by Labs 5 and 17).

STATISTICAL ANALYSIS

Data from each assay was used to calculate the relative potency of samples A, B, and C, relative to sample S, by parallel line analysis using the software program CombiStats (8). In this analysis log-transformed responses (i.e. clotting times) are plotted against log-transformed concentrations. Tests of validity (significance of non-linearity and non-parallelism) were performed at the 1% level ($p < 0.01$). Deviations from linearity or parallelism were investigated further. Non-parallelism was assessed by comparing the ratios of fitted slopes for the samples relative to the standard. A ratio between 0.90 – 1.11 was considered to confirm acceptable parallelism. Non-linearity was assessed by visual inspection of the plotted data, to rule out anomalously significant results due to tight replicates (and under-estimation of the residual error). Visual inspection of the plotted data also allowed identification of points outside the linear portion of the dose response, and the cause of non-linearity. If removal of these points improved linearity and resulted in a statistically valid assay ($p > 0.01$), the remaining three points were used for potency calculations, and the assay included in the analysis. It should be noted that in most cases excluding points in this manner has minimal effect on the overall potency determination but does improve statistical agreement between standards and test samples, as all assays in the study are judged by the same exclusion criteria.

Data from all valid assays were combined to generate unweighted geometric mean potencies for each laboratory and these laboratory means were then used to calculate an overall unweighted geometric mean for each sample, for each assay method. Comparisons between groups (methods, substrates) were performed with t-tests (unpaired, two-tailed, equal variance) or analysis of variance (ANOVA) using $\log_{10}$ transformed potency estimates in Minitab (version 18, Minitab Inc. USA).

Variability between assays and laboratories were expressed using geometric coefficients of variation (GCV = $\{10^{S}-1\}$ x 100%), where s is the standard deviation of the $\log_{10}$ transformed potencies) (9).

PARTICIPANTS

A total of 20 laboratories agreed to take part in the study. The majority of participants were users of the current IS, and so were familiar with carrying out thrombin potency assays. The participating laboratories were from a wide geographical area, including Austria (4), Germany (1), Belgium (1), Italy (1), Netherlands (2), Poland (1), Spain (1), USA (1), Australia (1), South Korea (2), India (1), Israel (1) and the UK (3). Of these, 9 were regulatory, 7 were industrial, and 4 were academic. Laboratories were randomly assigned a laboratory code at the outset of the study. The list of participants is provided in Appendix 2.

RESULTS

Assay deviations and exclusions

Assay exclusions and deviations are outlined below.

Laboratory 1 performed a three-point dose-response instead of four.

Laboratory 3, plasma plate clotting, the lowest dose was removed from all assays due to significant non-linearity

Laboratory 4 measured a single three-point dilution series in duplicate.

Laboratory 6 measured a single three-point dilution series in duplicate, except for sample S which had a four-point dilution series.

Laboratory 7, plate fibrinogen clotting, assay 2, the second replicate for sample B was removed due to a lack of doses response. For plasma plate clotting assays, the lowest dose was removed from all assays due to significant non-linearity

Laboratory 8, automated plasma clotting, the lowest dose was removed from all assays due to significant non-linearity. For chromogenic assays 3 and 4, the lowest dose was removed due to significant non-linearity.

Laboratory 11, automated fibrinogen clotting, performed a five-point dose-response. The highest dose was removed from all assays to improve linearity and parallelism.

Laboratory 12, plate fibrinogen clotting, the highest dose was removed from assays 1 and 4 due to significant non-linearity

Laboratory 14 did not return any results.

Laboratory 16, plate plasma clotting, assay data provided a better fit to the parallel line model (better correlation coefficient) if untransformed clotting times were used. For the automated plasma method, the lowest dose was removed from assay 1 to improve linearity.

Laboratory 17 performed a three-point dose-response instead of four.

Laboratory 18, plasma plate clotting, assay 2 was removed due to significant non-parallelism.

Laboratory 19 performed a three-point dose-response instead of four.

Laboratory 20, automated fibrinogen clotting, assays 1 and 2, untransformed clotting times were used to improve linearity and provide a better fit to the parallel line model.

Assay data

Table 1 shows a summary of methods and substrates used in the collaborative study. Nineteen laboratories contributed a total of 111 assays after exclusions, which comprised 91 clotting assays, 56 with fibrinogen and 35 with plasma. Of these, 52 were performed with automated coagulometers, 12 with manual coagulometers, and 27 with plate-based assays. Four laboratories performed chromogenic assays (16 assays in total) and one laboratory performed fluorogenic assays (4 assays).

Detailed values of individual laboratory mean potencies for sample A are shown in Table 2 and for samples B and C in Table 3. Potencies are calculated relative to sample S, the 2nd IS for Thrombin, along with 95 % confidence intervals for each laboratory estimate. Summary statistics for all samples and methods, are shown in Table 4.

Inter-and intra-laboratory variation

There was generally low variability within each laboratory for all samples, expressed as the intra-laboratory % GCV, which were under 5 % in most cases, indicating the laboratories performed the assays well. Variability between the laboratories, expressed as the inter-laboratory % GCV, was also low, and < 5 % for all methods and samples. Variability between laboratories was slightly lower for chromogenic assays compared to clotting assays for all samples, although fewer participants used this method. For clotting assays, intra- and inter-laboratory variability was slightly lower with fibrinogen as substrate compared to plasma, a trend also observed in the previous international collaborative study (4). Plate-based assays were slightly more variable than coagulometer methods.

Potency estimates of sample A versus sample S

The overall mean potency for sample A relative to sample S by clotting assays was 101.1 IU/ampoule, significantly lower than the mean potency from chromogenic assays of 111.5 IU/ampoule (Tables 2 and 4, $p < 0.005$). These values are in excellent agreement with potencies calculated for sample A relative to sample S in the previous collaborative study (102.8 IU/ampoule by clotting, and 111.7 IU/ ampoule by chromogenic) (4). Potency estimates by plasma clotting assays were slightly lower than fibrinogen clotting assays (98.8 vs 102.5 IU/ampoule, respectively), which was statistically significant ($p < 0.01$). Potency estimates from the fluorogenic assays were midway between clotting and chromogenic (106.5 IU/ampoule). Variation between and within laboratories was low (inter-lab GCV = 3.5 % by clotting, 1.8 % by chromogenic; mean intra-lab GCV = 2.8 % by clotting, and 4.4 % by chromogenic).

Potency estimates by laboratory and method are also displayed in the form of a histogram (Figure 1), which illustrates the very good agreement between the laboratories. Laboratory estimates are centred around 100 IU/ampoule, the mean potency by clotting assays. The higher potencies calculated by chromogenic assays are all clustered on the right of the plot.

Potency estimates of samples B and C versus sample S

Overall mean potency estimates by clotting assays for coded duplicate samples B and C were almost identical (90.4 and 90.3 IU/ampoule, respectively, Tables 3 and 4). Potencies obtained by chromogenic assays were in good agreement with the clotting assays, with overall mean potencies of 88.8 IU/ampoule for sample B and 87.5 IU/ampoule for sample C. The single lab estimates by fluorogenic assays were 89.4 and 90.5 IU/ampoule for samples B and C, respectively. The good agreement between estimates for samples B and C both within and between laboratories can be seen in Figure 2, which plots individual laboratory estimates with their 95 % confidence intervals.

As samples B and C are duplicate samples of the same preparation (19/188), the ratio of estimates should equal 1. As shown in Table 3, all laboratories obtained a ratio within 5 % of this value, and most laboratories were within 3 %. The geometric mean of all ratios was 1.00. Variability within each laboratory was generally very low, and below 5 % in most cases, which is reflected in the narrow 95 % confidence intervals for laboratory potency estimates in Figure 2.

Combined potency estimates of samples B + C versus sample S

As there was no difference between the datasets for samples B and C, and they are coded duplicates of a single candidate preparation (19/188), laboratory mean potencies were recalculated as the geometric mean of samples B and C in each assay, together with their % GCVs. Individual laboratory estimates are given in Table 5 and summary statistics in Table 4. The data are also shown in Figure 3 in histogram form. The potencies obtained from combining samples B and C were 90.4 IU/ampoule by clotting assays (inter-lab GCV = 3.9 %) and 88.1 IU/ampoule by chromogenic assays (inter-lab GCV = 2.5 %), which were not significantly different. The good agreement between laboratories and the assay methods are apparent from Figure 3, with the majority of laboratory estimates centred around 90 IU/ampoule, with no pattern or bias for any particular method. Fluorogenic assays gave a mean potency of 89.9 IU/ampoule, in good agreement with the other methods. Variability within the laboratories for the combined estimates of sample B and C was low (mean GCV = 3.5 % by clotting assays and 4.4 % by chromogenic assays) and comparable to the variability of sample A.

Clotting assays by method and substrate

A summary of the results obtained by clotting assays grouped according to method and substrate are presented in Table 6.

Potency estimates by plate-based clotting assays were slightly lower than estimates by automated and manual coagulometers, and slightly more variable. However, potency estimates by the various clotting methods were not significantly different for any of the samples. Mean intra-laboratory GCVs by plate assays were 5.7 % and 5.3 % for samples A and sample B/C respectively, compared to 2.2 % and 3.4 % for automated assays. The higher variability of plate assays might reflect the greater number of pipetting steps required for plate assays and the use of multichannel pipettes, compared to precisely timed robotic liquid handling systems in automated coagulometers. However, variability between laboratories performing plate clotting assays was still low (GCV = 3.5 % and 4.9 % for samples A and B/C respectively), and comparable to the automated assays (GCV = 3.5 % and 2.9 % for samples A and B/C respectively). Potency estimates obtained by manual coagulometers, which measure mechanical properties of the clot, were very similar to turbidimetric clotting methods for all samples, although they were performed by fewer laboratories.

Potency estimates by plasma clotting assays were slightly lower than fibrinogen clotting assays, which was statistically significant for sample A but not for sample B/C. Plasma clotting assays also had slightly higher intra- and inter-laboratory GCVs than fibrinogen. However, these values were still low, and comparable to the previous collaborative study, which also noted slightly lower potency estimates and higher variability by plasma clotting compared to fibrinogen clotting (4).

Modelling of long-term stability

Accelerated degradation testing has been performed to investigate the long-term stability of the candidate standards. Ampoules are stored at a range of temperatures (-20 °C, +4 °C, +20 °C, +37 °C, +45 °C, and +56 °C) and potency measurements at the higher temperatures relative to the lower temperatures after prolonged periods are used to fit the Arrhenius model (which relates the rate of decay to temperature). Using this model, it is possible to predict the stability of the standards over a period of years when stored at -20 °C (10).

Prior to the current study, stability modelling was performed on the 2nd IS (sample S, 01/580) and sample A (01/578) after 13 years storage at elevated temperatures. The potency of samples stored at each temperature were determined by fibrinogen clotting assays with a manual coagulometer, relative to samples stored at -20 °C (Table 7). These data were used to fit the Arrhenius equation and extrapolate the predicted % potency loss per year at -20 °C, which is < 0.01 % for both samples, indicating our freeze-dried thrombin preparations have excellent stability profiles.

To obtain an estimate of the long-term stability of sample B/C (19/188), ampoules were stored at elevated temperatures for 6 months, and the potencies determined by chromogenic and automated fibrinogen clotting assays, relative to samples stored at -20 °C (Table 8). The data provided a good fit to the Arrhenius model, with predicted potency losses of 0.036 % and 0.041 % per year by clotting and chromogenic assay, respectively. These early estimates indicate that sample B/C (19/188) is very stable, and it is likely the accuracy and precision of the long-term stability estimates will improve as the duration of the accelerated degradation study increases. The source of thrombin and the formulation used to fill 19/188 is the same as 01/580, giving us every reason to expect 19/188 will have an excellent long-term stability profile, similar to 01/580. A number of ampoules of 19/188 remain in storage at elevated temperatures for ongoing stability monitoring with chromogenic and clotting methods.

Bench stability following reconstitution

We investigated the stability of sample B/C (19/188) following reconstitution, to replicate conditions experienced during a routine assay period. Reconstituted material was transferred to stoppered tubes and placed on melting ice for an entire working day (8 hours) or overnight (24 hours), and potencies determined relative to a freshly reconstituted ampoule using fibrinogen clotting assays in a microtitre plate format (Appendix 1). The results in Table 9 show that sample B/C (19/188) is very stable after reconstitution, with little loss in activity over the assay period.

PROPOSAL

It is proposed that 19/188 is adopted as the WHO 3rd IS for Thrombin, with a potency of 90 IU per ampoule based on combined clotting assays from samples B and C. The good

agreement between clotting and chromogenic assays for sample B/C suggests a higher α-thrombin content compared to sample A (01/578). There was no difference between estimates by fibrinogen and plasma-based assays, or by plate, automated, and manual coagulometer clotting methods. Overall mean intra- and inter-laboratory % GCVs were low and similar for all methods, suggesting the standard would be suitable for use in a variety of assay formats.

Participants response to the study

A copy of the study report was circulated to the participants together with a Participants' Response Sheet (Appendix 3). All of the participants responded, and all agreed with the proposal of 19/118 as the 3rd IS for Thrombin with a potency of 90 IU per ampoule. Comments were generally complimentary, with one laboratory noting *"The report reads really very well, is clearly structured with excellent tables and figures, and makes a strong and clear case for the new standard(s)"*. There was only one comment from the participants which required addressing: *"We recommend adding a discussion or justification of the rules used to remove some participants' data for the lowest or the highest dose. If possible, it may be helpful to comment on the impact of data removal on the study conclusions in the case of each laboratory."* In response to this point we have elaborated on the criteria used for assay validity, and in particular the rationale for removing datapoints due to significant non-linearity, in the *Statistical Analysis* section of the study report.

Expert review by the ISTH-SSC

The study report was distributed to experts of the Scientific and Standardization Committee (SSC) of the International Society on Thrombosis and Haemostasis (ISTH) by Prof. Joost Meijers, SSC/ISTH-WHO Liaison Officer, for further review during June 2020. Responses were received by 10 experts and all agreed with the proposal, which was formally endorsed at the SSC Executive Board meeting on 9th July 2020.

The following points were raised by the SSC experts:

Expert 1:

The top of page 5 mentions 'clot lysis times', which is likely an error. Although it is reported that 19 labs returned results, 2 labs had the same PI. Were different methods used by the two different labs of the same PI?

The expert is correct, this was an error, and "clot lysis times" has been replaced with "clotting times". Regarding the two laboratories with the same PI, the same methods were used by the different laboratories with different technical staff. However, all assays were performed independently from each other, using different sets of study materials. Participants were free to choose their own methods and substrates for the assays, and it is not something we wanted to influence.

Expert 2:

The study was well executed, the report is clear and transparent, and the results are convincing, - for me an excellent piece of work and I have nothing to add.

Expert 3:

No comments.

Expert 4:

Why not provide analysis of the starting material (biochemistry: purity, SDS Page, specific activity etc)?

We do not usually provide this kind of characterisation because the important factor is the end product after formulation and freeze drying and the collaborative study is the process we use to find out what is in the ampoules, in terms of biological activity. Our formulations of WHO standards usually contain albumin as a stabiliser and this kind of information can lead to misunderstanding around the nature of our standards.

Page 5: "it was clear from the plotted data", relatively weak description, especially if that leads to exclusion of values.

We have now re-phrased this sentence to make it clear what aspects of the plotted data we are referring to: "Visual inspection of the plotted data also allowed identification of points outside the linear portion of the dose response, and the cause of non-linearity."

Nice variation in methods and labs!

Page 8: very strong that duplicate samples gave the same results.

Page 10, bottom: in coagulation diagnostics, melting ice is not used anymore. Data on bench stability are correct, but are less useful for current daily practice.

Participants in the study were requested to reconstitute ampoules and keep them on ice while they performed the assays. We check that there would be no deterioration in activity over the couple of hours that the assays take. We are happy to report that there all samples are stable on ice. Where users have different methods for handling or storing our standards we always suggest they investigate stability under their own conditions. We cannot make recommendations for all conditions employed by end-users of our standards.

Page 26: freeze dried fibrinogen usually contains salt and should be dissolved in water. Was buffer used here? Dilution will then be in different buffers, that therefore may result in differences in final concentration of components of the buffer. Although this most likely will not influence the results, the question is why this was done?

We've found that reconstitution of fibrinogen to the high stock concentrations we favour is improved using buffer rather than water. Since the diluent used for the assays was also HEPES-based, we saw no reason to not dissolve the fibrinogen in this buffer from the outset. These were only guidelines, and participants were free to prepare their fibrinogen stocks as they saw fit. As mentioned by the expert, given that the fibrinogen stock is diluted approximately 25-50-fold, it is unlikely to influence the results.

Expert 5:

One comment with respect to the report.
Page 7, paragraph Assay Data, first sentence.
Table 1 shows a summary of assay results grouped by method and substrate.
This sentence is not correct and should be replaced by:
Table 1 shows a summary of methods and substrates used in this collaborative study.

We have now changed this sentence in the report.

Expert 6:

Well conducted study with sufficient participating institutes, adequate assays with enough variation in methodology. The report is well written and conclusions are supported by the provided data.

Expert 7:

This study is finely executed, and the paper is very well written.
My only minor comment is on the removal of some participant's data for the lowest or the highest dose. I very much agree with the comment of the participant: "If possible, it may be helpful to comment on the impact of data removal on the study conclusions in the case of each laboratory".
The authors' response was the following: In response to this point we have elaborated on the criteria used for assay validity, and in particular the rationale for removing datapoints due to significant non-linearity. The addition of these methodological issues to the Statistical Analysis section of the study report is valid and the need for this is clear. Still, one wonders about the extent that results became different after data removal for each laboratory- is it substantial or not?

We should be clear about why we remove data points in the first place, as this seems to have caused confusion.

For the parallel-line model to be valid for potency calculations, the test samples must be both linear and parallel with the standard to which they are being compared. If this is not the case, the potency estimates from the assay are not statistically valid, and the potency estimates cannot be used in the study. At this point we have a choice – discard the entire assay or scrutinise the data further to try and identify the root cause of the non-linearity.

We first examine the plotted data (log-transformed responses plotted against log-transformed concentrations). From this, it is sometimes apparent that the cause of the non-linearity is the highest or lowest dose falling outside the linear range (the straight lines will 'kink' at one end of the dose response curve). If this is the case, we test whether removal of these points produces an assay that is not significantly non-linear. If so, the assay is now considered valid, and the potency estimates are included in the study. This approach allows us to keep as much good data as possible and obtain statistically valid potency estimates.

With this in mind, and considering the study data as a whole, it is therefore not a case of having two sets of data with and without exclusions, as we cannot include invalid assays in our analysis in the first place.

Our approach has been followed for many years and in many international collaborative studies. All exclusions and assay deviations are reviewed and approved by our statisticians.

Expert 8:

Well executed study. Fully support the new standard.

Expert 9:

No comments.

Expert 10:

I concur with the comment from one of the testing laboratories:

"The report reads really very well, is clearly structured with excellent tables and figures, and makes a strong and clear case for the new standard(s)".

INSTRUCTIONS FOR USE

A draft Instructions for Use to accompany 19/188 is provided in Appendix 4.

ACKNOWLEDGEMENTS

We are extremely grateful to Dr Sabine Fraiss and colleagues at Takeda (Vienna, Austria) for the donation of α-thrombin used to prepare the new candidate International Standard. We are also grateful to the laboratories and personnel who gave their time and took part in the study to provide the data presented in this report (participants are listed in Appendix 2).

The FXIII and Fibrinogen SSC Subcommittee of the ISTH.

The team from the Centre for Biological Reference Materials (CBRM) at NIBSC.

The team from the Centre for AIDS Reagents (CFAR) at NIBSC for shipping the study materials.

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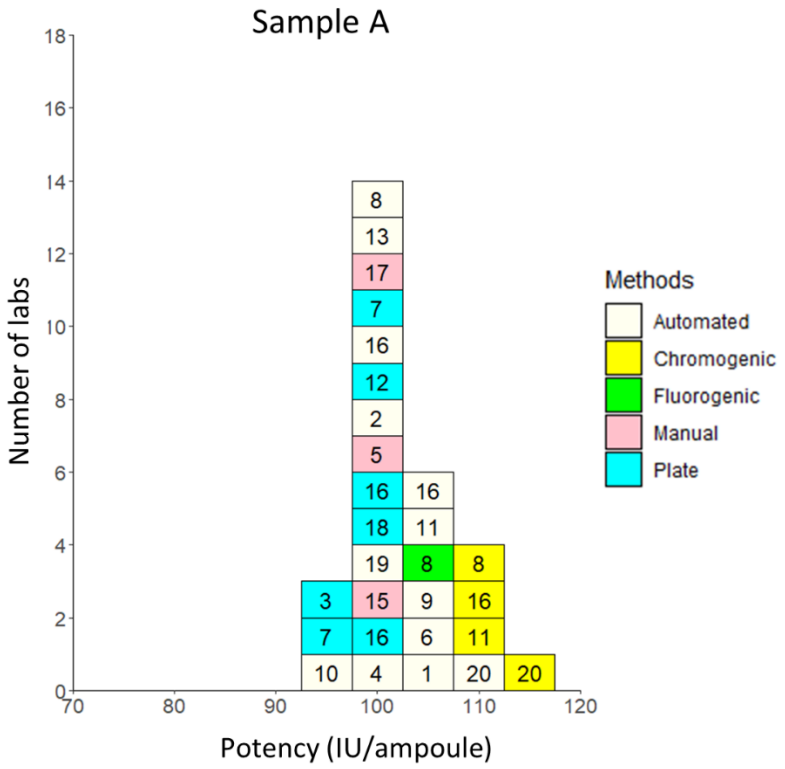


Figure 1. Histogram summarising potency estimates of sample A relative to sample S, the 2nd IS for Thrombin (01/580).
Each box represents the geometric mean potency estimate (IU/ampoule) from the laboratory coded by the number in the box. The y-axis is the number of laboratories with results in the corresponding concentration range, and the colours represents the different assay methods used.

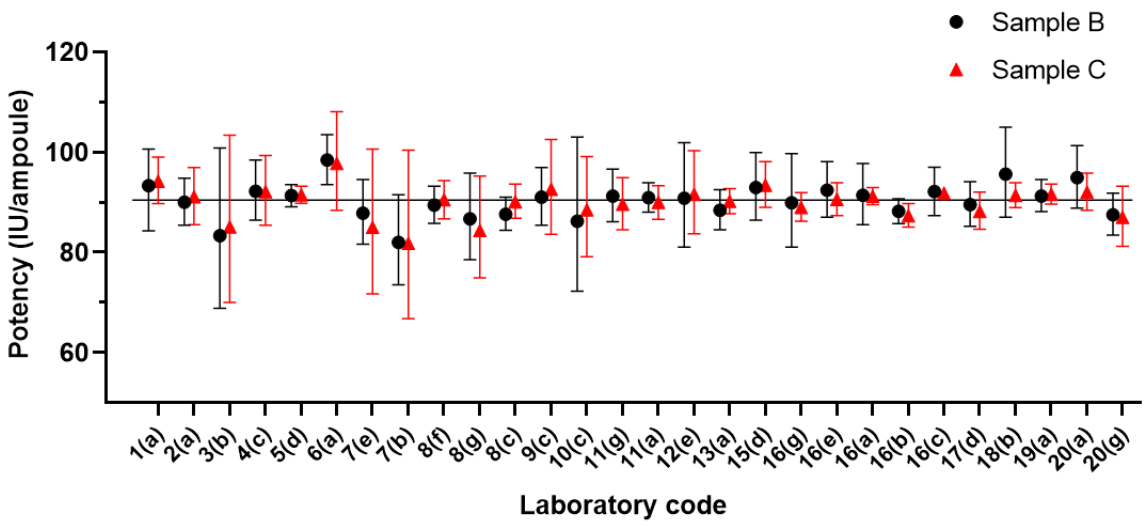


Figure 2. Potency estimates for coded duplicate samples B and C (19/188) relative to sample S, the 2nd IS for Thrombin (01/580). Each point represents the geometric mean potency for each laboratory. Error bars are 95 % confidence intervals of the mean. The line at 90 IU/ampoule is the final potency assigned to the combined potency estimates from samples B/C (19/188) by clotting assays and proposed as the 3rd IS for Thrombin. Letters next to the laboratory code refer to the method(s) performed by each laboratory:
(a) automated clotting fibrinogen, (b) plate clotting plasma, (c) automated clotting plasma, (d) manual clotting fibrinogen, (e) plate clotting fibrinogen, (f) fluorogenic, (g) chromogenic.

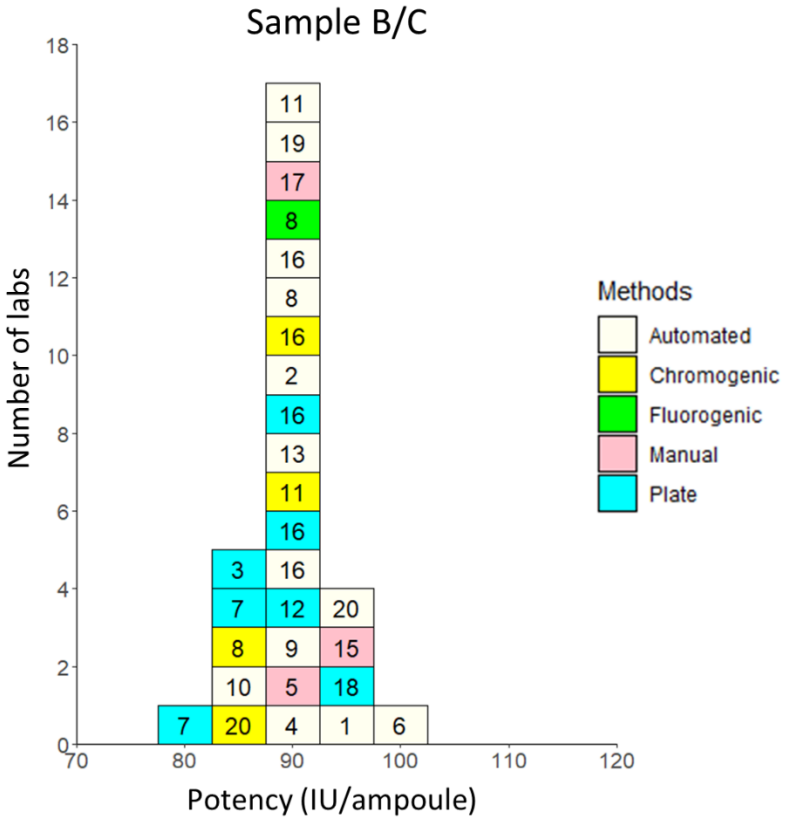


Figure 3. Histograms summarising potency estimates of combined samples B/C relative to sample S, the 2nd IS for Thrombin (01/580).
Each box represents the geometric mean potency estimate (IU/ampoule) from the laboratory coded by the number in the box. The y-axis is the number of laboratories with results in the corresponding concentration range, and the colours represents the different assay methods used.

Table 1. Summary of methods and substrates used in the collaborative study.
The number of laboratory estimates for each method and substrate are given, with numbers of assays contributed to the study in brackets.

		No. of laboratory estimates (or assays)			
Assay Method	**Substrate**	**Automated coagulometer**	**Manual coagulometer**	**Plate**	**Total**
Clotting	Fibrinogen	8 (32 assays)	3 (12 assays)	3 (12 assays)	14 (56 assays)
	Plasma	5 (20 assays)	Nil	4 (15 assays)	9 (35 assays)
Chromogenic					4 (16 assays)
Fluorogenic					1 (4 assays)
					28 (111 assays)

Table 2. Potency estimates for sample A (01/578) relative to sample S, the 2nd IS for Thrombin (01/580).

Method	Lab code	Substrate	No. of assays	Mean potency (IU/ampoule)	95% CI	Intra-lab GCV %
Automated clotting	1	Fibrinogen	4	105.8	102.3-112.2	3.2
	2	Fibrinogen	4	101.5	96.3-107.0	3.4
	4	Plasma	4	102.2	99.8-104.7	1.5
	6	Fibrinogen	4	105.3	98.4-112.6	4.3
	8	Plasma	4	98.7	96.0-101.6	1.8
	9	Plasma	4	104.7	98.7-111.1	3.8
	10	Plasma	4	94.7	90.9-98.7	2.6
	11	Fibrinogen	4	102.6	100.9-104.3	1.0
	13	Fibrinogen	4	99.9	98.0-101.8	1.2
	16	Fibrinogen	4	102.6	99.9-105.3	1.7
	16	Plasma	4	101.7	100.6-102.9	0.7
	19	Fibrinogen	4	100.3	95.1-105.8	3.4
	20	Fibrinogen	4	108.1	99.2-117.8	5.6
Plate clotting	3	Plasma	4	92.8	76.0-113.3	13.4
	7	Fibrinogen	4	101.4	81-4-126.4	14.8
	7	Plasma	4	96.7	82.4-113.4	10.6
	12	Fibrinogen	4	101.1	94.7-108.0	4.2
	16	Fibrinogen	4	102.5	99.7-105.5	1.8
	16	Plasma	4	98.4	91.0-106.3	5.0
	18	Plasma	3	100.2	94.1-106.7	2.6
Manual clotting	5	Fibrinogen	4	101.2	99.2-103.2	1.3
	15	Fibrinogen	4	101.2	99.3-103.1	1.2
	17	Fibrinogen	4	102.2	99.2-105.3	1.9
Chromogenic	8	S-2238	4	109.0	96.9-122.5	7.7
	11	In-house	4	111.9	106.9-117.1	2.9
	16	S-2238	4	111.6	105.8-117.8	3.4
	20	CS-01(38)	4	113.6	105.0-123.0	5.1
Plate fluorogenic	8	Z-Gly-Gly-Arg-AMC	4	106.5	104.1-109.0	1.5

Table 3. Potency estimates for coded duplicate samples B and C (19/188) relative to sample S, the 2nd IS for Thrombin (01/580).

Method	Lab code	Substrate	No. of assays	Sample B			Sample C			Ratio B/C
				Mean	95% CI	Intra-lab GCV %	Mean	95% CI	Intra-lab GCV %	
Automated clotting	1	Fbgn	4	93.3	84.3-100.6	6.8	94.2	89.7-99.0	3.2	0.99
	2	Fbgn	4	90.0	85.4-94.8	3.3	91.1	85.5-96.9	4.0	0.99
	4	Plasma	4	92.2	86.4-98.4	4.2	92.1	85.4-99.3	4.9	1.00
	6	Fbgn	4	98.4	93.5-103.5	3.3	97.8	88.4-108.1	6.5	1.01
	8	Plasma	4	87.6	84.4-91.0	2.4	90.1	86.8-93.6	2.4	0.97
	9	Plasma	4	91.0	85.4-96.9	4.1	92.6	83.6-102.5	6.6	0.98
	10	Plasma	4	86.2	72.2-103.0	11.8	88.5	79.1-99.1	7.3	0.97
	11	Fbgn	4	90.9	88.0-93.9	2.1	90.0	86.6-93.3	2.3	1.01
	13	Fbgn	4	88.4	84.5-92.5	2.9	90.2	87.7-92.7	1.7	0.98
	16	Fbgn	4	91.4	85.5-97.7	4.3	91.2	89.5-92.9	1.2	1.00
	16	Plasma	4	92.1	87.3-97.0	3.4	91.8	91.0-92.5	0.5	1.00
	19	Fbgn	4	91.2	88.1-94.5	2.2	91.6	89.6-93.6	1.4	1.00
	20	Fbgn	4	94.9	88.8-101.3	4.2	92.0	88.4-95.8	2.6	1.03
Plate clotting	3	Plasma	4	83.3	68.8-100.8	12.7	85.1	70.0-103.4	13.0	0.98
	7	Fbgn	4	87.8	81.6-94.5	4.7	85.0	71.7-100.6	11.3	1.03
	7	Plasma	4	82.0	73.5-91.5	7.1	81.8	66.7-100.4	13.7	1.00
	12	Fbgn	4	90.8	81.0-101.9	7.5	91.6	83.7-100.3	5.9	0.99
	16	Fbgn	4	92.4	87.0-98.1	3.8	90.6	87.3-93.9	2.3	1.02
	16	Plasma	4	88.2	85.7-90.7	1.7	87.3	85.0-89.7	1.7	1.01
	18	Plasma	3	95.6	87.0-105.0	3.9	91.4	88.9-93.9	1.1	1.05
Manual clotting	5	Fbgn	4	91.3	89.1-93.5	1.5	91.5	89.8-93.2	1.2	1.00
	15	Fbgn	4	92.9	86.4-99.9	4.7	93.4	89.0-98.1	3.1	0.99
	17	Fbgn	4	89.5	85.2-94.1	3.2	88.2	84.6-92.0	2.7	1.02
Chromogenic	8	S-2238	4	86.7	78.5-95.8	6.5	84.4	74.9-95.2	7.8	1.03
	11	In-house	4	91.2	86.1-96.6	3.7	89.6	84.5-94.9	3.7	1.02
	16	S-2238	4	89.9	81.0-99.7	6.7	89.0	86.2-91.9	2.0	1.01
	20	CS-01(38)	4	87.5	83.4-91.8	3.0	87.0	81.2-93.2	4.4	1.01
Plate fluorogenic	8	Z-Gly-Gly-Arg-AMC	4	89.4	85.8-93.2	2.7	90.5	86.7-94.3	2.7	0.99

Table 4. Summary statistics of samples A, B, C, and combined samples B/C, relative to sample S, the 2nd IS for Thrombin (01/580).

Sample	Assay method	Substrate	No. of Assays	Mean Potency (IU/ampoule)	Mean intra-laboratory variability (%)	Inter-laboratory variability (%)
A	Clotting	Fbgn	56	102.5	2.6	2.3
		Plasma	35	98.8	3.2	3.9
		Overall	91	101.1	2.8	3.5
	Chromogenic		16	111.5	4.4	1.8
	Fluorogenic		4	106.5	1.5	N/A
B	Clotting	Fbgn	56	91.6	3.6	2.9
		Plasma	35	88.6	4.6	5.3
		Overall	91	90.4	3.9	4.2
	Chromogenic		16	88.8	4.7	2.4
	Fluorogenic		4	89.4	2.7	N/A
C	Clotting	Fbgn	56	91.3	2.8	3.2
		Plasma	35	88.9	3.6	4.3
		Overall	91	90.3	3.1	3.8
	Chromogenic		16	87.5	4.0	2.7
	Fluorogenic		4	90.5	2.7	N/A
B/C	Clotting	Fbgn	56	91.4	3.3	3.0
		Plasma	35	88.7	4.6	4.6
		Overall	91	90.4	3.5	3.9
	Chromogenic		16	88.1	4.4	2.5
	Fluorogenic		4	89.9	2.5	N/A

Table 5. Combined potencies of samples B and C relative to sample S, the 2nd IS for Thrombin (01/580). Geometric mean potencies and GCVs are calculated for each laboratory by combining assays for samples B and C.

Method	Lab code	Substrate	No. of assays	Mean potency	Intra-lab GCV %
Automated clotting	1	Fibrinogen	4	93.7	4.9
	2	Fibrinogen	4	90.5	3.5
	4	Plasma	4	92.1	4.2
	6	Fibrinogen	4	98.1	4.7
	8	Plasma	4	88.9	2.7
	9	Plasma	4	91.8	5.2
	10	Plasma	4	87.4	9.2
	11	Fibrinogen	4	90.5	2.1
	13	Fibrinogen	4	89.3	2.4
	16	Fibrinogen	4	91.3	2.9
	16	Plasma	4	91.9	2.2
	19	Fibrinogen	4	91.4	1.7
	20	Fibrinogen	4	93.4	3.6
Plate clotting	3	Plasma	4	84.2	11.9
	7	Fibrinogen	4	86.4	8.1
	7	Plasma	4	81.9	10.0
	12	Fibrinogen	4	91.2	6.2
	16	Fibrinogen	4	91.5	3.1
	16	Plasma	4	87.8	1.7
	18	Plasma	3	93.5	3.6
Manual clotting	5	Fibrinogen	4	91.4	1.3
	15	Fibrinogen	4	93.2	3.7
	17	Fibrinogen	4	88.9	2.8
Chromogenic	8	S-2238	4	85.6	6.8
	11	In-house	4	90.4	3.6
	16	S-2238	4	89.4	4.6
	20	CS-01(38)	4	87.3	3.5
Plate fluorogenic	8	Z-Gly-Gly-Arg-AMC	4	89.9	2.5

Table 6. Summary statistics for clotting assays according to substrate (plasma or fibrinogen) and method (automated/manual coagulometer or plate assay), for samples A and B/C, relative to sample S.

Substrate		Sample A				Sample B/C			
		Auto	Plate	Manual	Overall	Auto	Plate	Manual	Overall
Plasma	Mean potency (IU/ampoule)	100.3	97.0	-	**98.8**	90.4	86.7	-	**88.7**
	Mean intra-lab GCV %	1.8	6.5	-	**3.2**	4.1	5.2	-	**4.6**
	Inter-lab GCV %	3.9	3.3	-	**3.9**	2.4	5.9	-	**4.6**
	No. of labs	5	4	-	**9**	5	4	-	**9**
Fibrinogen	Mean potency (IU/ampoule)	103.2	101.8	101.5	**102.5**	92.2	91.4	91.1	**91.4**
	Mean intra-lab GCV %	2.6	4.8	1.4	**2.6**	3.0	5.4	2.4	**3.3**
	Inter-lab GCV %	2.8	0.7	0.6	**2.3**	3.0	3.3	2.4	**3.0**
	No. of labs	8	3	3	**14**	8	3	3	**14**
Overall	Mean potency (IU/ampoule)	102.1	99.0	101.5	**101.1**	91.5	88.0	91.1	**90.4**
	Mean intra-lab GCV %	2.2	5.7	1.4	**2.8**	3.4	5.3	2.4	**3.5**
	Inter-lab GCV %	3.5	3.5	0.6	**3.5**	2.9	4.9	2.4	**3.9**
	No. of labs	13	7	3	**23**	13	7	3	**23**

Table 7. Potency remaining for samples S (01/580) and A (01/578) after 13 years storage at elevated temperatures, relative to ampoules stored at -20 °C. Results are based on a combined potency from two ampoules, each assayed in duplicate. Potencies were determined by fibrinogen clotting assays using a manual coagulometer (KC4 delta, Tcoag/Diagnostica Stago, Theale, UK).

Storage temperature (°C)	% potency remaining relative to -20 °C			
	01/578		01/580	
	Mean	95% CI	Mean	95% CI
+4	98.8	94.9-102.9	99.0	93.5-104.9
+20	95.5	89.1-102.4	94.8	92.1-97.6
+37	78.8	73.2-67.8	79.2	74.5-84.0
+45	65.1	62.5-67.8	67.2	65.2-69.2
Predicted loss per year (%)	0.007		0.009	
Upper 95% CI of potency loss (%)	0.047		0.039	

Table 8. Potency remaining for sample B/C (19/188) after 6 months storage at elevated temperatures, relative to ampoules stored at -20 °C. Each result is based on a combined potency from two ampoules, each assayed twice in duplicate. Assays were performed using automated fibrinogen clotting and chromogenic microtitre plate methods described in the study protocol (Appendix 1).

Storage temperature (°C)	% potency remaining relative to -20 °C			
	Fibrinogen clotting		Chromogenic	
	Mean	95% CI	Mean	95% CI
+4	99.1	96.2-102.0	98.6	96.4-100.8
+20	99.2	95.9-102.6	99.4	95.7-103.3
+37	97.1	93.8-100.5	97.5	93.4-101.8
+45	92.2	89.0-95.6	96.4	94.3-98.6
+56	85.6	83.1-88.2	94.0	92.0-96.1
Predicted loss per year (%)	0.036		0.041	
Upper 95% CI of potency loss (%)	0.619		1.588	

Table 9. Bench stability following reconstitution. Ampoules of sample B/C (19/188) were reconstituted and incubated on melting ice for 8 or 24 h. Potencies were determined relative to a freshly-reconstituted ampoule by fibrinogen clotting assays, using the microtitre plate clotting assay guidelines in Appendix 1.

Time following reconstitution at 4°C	% activity remaining (relative to freshly-opened ampoule)	
	Sample B/C (19/188)	95% CI
8 h	96.9	91.4-102.7

24 h	99.3	93.7-105.3

Appendix 1. Study protocol.

International Collaborative Study to Establish the WHO 3rd IS for Thrombin

Study protocol CS656

1. SAMPLES PROVIDED FOR ASSAYS

4 ampoules of each of the following samples are provided for use in at least 4 independent assays:

S WHO 2nd International Standard for Thrombin (01/580), 110 IU/ampoule

A Candidate test material, ~ 100 IU/ampoule

B Candidate test material, ~ 90 IU/ampoule

C Candidate test material, ~ 90 IU/ampoule

Further information, including health and safety data, is available in the instructions for use documents provided with the samples.

Laboratories performing assays using more than one method are requested to perform all methods using the same ampoule sets, if possible. Further ampoules can be made available on request.

2. STORAGE AND RECONSTITUTION OF SAMPLES S, A, B AND C

Four ampoules of each sample Samples S, A, B and C are shipped at ambient temperature. Store unopened ampoules at -20°C or below. Immediately before beginning an assay allow the ampoules to warm to room temperature before reconstitution for approximately 30 min. Ensure that all of the contents are in the lower part of the ampoule by gently tapping. Open the ampoules as directed below and reconstitute by adding 1.0 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 stoppered plastic tubes and store on ice during the assay period.

Directions for opening ampoules
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 (labelled) 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.

3. STUDY PLAN, ASSAY METHOD AND DESIGN

Please use your own assay method if one is available, adapting it to the study requirements described below. The attached document **"Guidelines on clotting and chromogenic assays to measure Thrombin"** provides example methods. The example methods have been designed so that plate methods with plasma, fibrinogen, and chromogenic assays can use the same buffers and thrombin dilution series to facilitate use of multiple methods. If you want to repeat the study using more than one method, we encourage you to do so.

You are requested to carry out 4 independent assays (Assay 1-4), each using one set of fresh ampoules of S, A, B, and C. For each assay, two independent dilution series from each ampoule should be prepared. The dilution range should lie in the linear region of the dose-response relationship.

A balanced order of testing should be followed when preparing the samples. For example, to avoid systematic errors due to dilution errors or plate effects you should vary the arrangement of samples on the plate or coagulometer. For example:

Day/session 1	**Assay 1**	**S**	**A**	**B**	**C**	**C'**	**B'**	**A'**	**S'**
	Assay 2	**A**	**B**	**C**	**S**	**S'**	**C'**	**B'**	**A'**
Day/session 2	**Assay 3**	**B**	**C**	**S**	**A**	**A'**	**S'**	**C'**	**B'**
	Assay 4	**C**	**S**	**A**	**B**	**B'**	**A'**	**S'**	**C'**

Statistical analysis requires a dilution range of at least 3 doses each with 2 replicate readings. Each letter (S, A, B and C) refers to a set of ≥ 3 different dilutions, and S', A', B' and C' refer to replicate sets of dilutions made independently from the same ampoule. Assays should be completed within 4 hours of sample reconstitution.

4. RESULTS

Please return the raw data from your assays by e-mail to matthew.locke@nibsc.org and colin.longstaff@nibsc.org by **31st January 2020**. If you need more time to complete the assays, please let us know.

To calculate the potency of the test samples relative to the IS 01/580 raw data are required. Ideally we would like to receive time courses of absorbance changes if available.

Acceptable data formats for clotting assays are:

I. Clotting time (i.e. time to a particular OD change, or time to 50 % clotting) in all wells or tubes (including all replicates, and not just the means).
II. Raw data in the form of clotting profiles (a column of time versus columns of absorbance data) in txt, csv, or xlsx format, for example.
III. A Softmax Data file (.pda or .sda).

Acceptable data formats for chromogenic assays are:

I. Rates of initial absorbance changes. These should be expressed as absorbance change per time (e.g. Abs/sec), or time to a particular OD change.
II. Raw data in the form of a kinetic profile (a column of time versus columns of absorbance data) in txt, csv, or xlxs format, for example.
III. A Softmax Data file (.pda or .sda).

If you have any further questions about what data to return, or acceptable formats, please contact matthew.locke@nibsc.org or colin.longstaff@nibsc.org.

Results sheets are provided for you to complete, or to use as an example when returning data in a different format (e.g. Excel). **We request you provide raw data for Thrombin concentration used in nominal IU/ml and responses. Calculation of potency is optional, as this will be carried out at NIBSC.** We will be happy to compare and discuss your calculations if you wish.

Data Analysis using Apps

Calculation of clotting time and rates of reaction can be facilitated using recently developed Apps, to facilitate reproducible data analysis.

For clot lysis assay data: https://drclongstaff.shinyapps.io/Clot_or_HaloCL
For chromogenic assay data: https://drclongstaff.shinyapps.io/zymogenactnCL/
Links to more detailed help files are available: https://drclongstaff.github.io/shiny-clots/

If you have any questions at all about the study, assay methods or reporting of results please do not hesitate to contact us.

Guidelines on clotting and chromogenic assays to measure Thrombin

MATERIALS

Buffers

Buffer A: **10 mM HEPES pH 7.4**

- 2.38 g/L HEPES, adjust to pH 7.4 with approx. 4 ml/L 1M NaOH. Solution is stable at 4 °C for several months.

Buffer B: **10 mM HEPES pH 7.4, 150 mM NaCl, 0.01 % tween**

- 2.38 g/L HEPES, 8.77 g/L NaCl, 1 ml/L 10 % tween-20, adjust to pH 7.4 with approx. 4 ml/L 1M NaOH. Solution is stable at 4 °C for several months.

Buffer C: **10 mM HEPES pH 7.4, 150 mM NaCl, 0.01 % tween, 40 mM $CaCl_2$, 1 mg/ml albumin**

(i.e. Buffer B + 40 mM $CaCl_2$ + 1 mg/ml human (or bovine) albumin)

- 2.38 g/L HEPES, 8.77 g/L NaCl, 40 ml/L 1 M $CaCl_2$, 1 ml/L 10 % tween-20, adjust to pH 7.4 with approx. 4 ml/L 1M NaOH. Solution is stable at 4 °C for several months.
- Add 500 µl 20 % (200 mg/ml) albumin to every 100 ml of buffer to make working buffer C. Prepare fresh on day of use.

Fibrinogen stock solution

One bottle of 0.5 g fibrinogen (from Calbiochem, for example) is dissolved in 10 ml of buffer A at RT to make a 50 mg/ml stock. The contents are stirred gently for 30 mins then dispensed as 0.5 ml aliquots in Eppendorfs and flash frozen before storage at -40 °C.

Human Plasma Stock Solution

Lyophilised or fresh/frozen plasma are suitable substrates which should be freshly reconstituted or thawed for each assay.

Chromogenic substrate stock solution

For example, S-2238 (Chromogenix) dissolved in distilled water to make a 3mM stock solution. Store at 4°C.

Thrombin standard and candidates

Ampoules S, A, B, and C should be reconstituted in 1 ml distilled water (described in the "Study Protocol").

S = WHO 2nd International Standard for Thrombin (01/580), 110 IU/ml

A = Candidate test material, ~ 100 IU/ml

B = Candidate test material, ~ 90 IU/ml

C = Candidate test material, ~ 90 IU/ml

Example of plate clotting assay used at NIBSC

ASSAY PRINCIPLE

Fibrinogen or plasma is clotted with a dilution range of thrombin in a microtitre plate, and clotting monitored by changes in turbidity using a plate reader. The time to half maximal absorbance is used as the time to 50 % clotting, which is inversely proportional to the amount of thrombin in the clotting mixture.

The aim of the study is to assign potencies to three candidate samples (labelled A, B, and C). The potencies of the test samples are determined by comparison with a reference preparation, in this case the WHO 2nd International Standard for Thrombin (code 01/580, Sample S), with an assigned potency of 110 IU per ampoule.

PREPARATION OF WORKING REAGENTS

Fibrinogen stock solution

A 4 mg/ml fibrinogen **working stock** is prepared by adding 320 µl of 50 mg/ml fibrinogen stock solution (thawed from frozen in a 37 °C water bath) to 3.68 ml buffer B. Maintain working stock at 37 °C.

Plasma working solution

If plasma is being used instead of fibrinogen, a plasma **working stock** is prepared by diluting plasma 1:1 with buffer B. Maintain working stock at 37 °C.

METHOD

Thrombin dilutions

Each assay requires at least two replicate dilution ranges of 4 doses of thrombin from one ampoule of S, A, B, and C. All dilutions are carried out in Buffer C. The most concentrated solution is 0.5 IU/ml and a range of doubling dilutions is prepared from this solution. Two pre-dilutions may be performed to make the 0.5 IU/ml solution as follows:

Sample	1st pre-dilution	2nd pre-dilution	Nominal concentration (IU/ml)
S	45 µl -> 1 ml	100 µl -> 1 ml	0.5 IU/ml
A	50 µl -> 1 ml	100 µl -> 1 ml	0.5 IU/ml
B	55 µl -> 1 ml	100 µl -> 1 ml	0.5 IU/ml
C	55 µl -> 1 ml	100 µl -> 1 ml	0.5 IU/ml

The 0.5 IU/ml dilution (designated D1) is then used to make three additional doubling dilutions (D2-D4) in Buffer C, using a multi-channel pipette in a 96-well plate (as shown in the example of a 96-well plate below, in blue).

S', A', B' and C' refer to replicate sets of dilutions (and pre-dilutions) made independently from the same ampoule.

96-well plate layout for serial dilutions

Sample ID:		**S**	**A**	**B**	**C**	**C'**	**B'**	**A'**	**S'**			
	1	2	3	4	5	6	7	8	9	10	11	12
A		D1	D1	D1	D1	D1	D1	D1	D1			
B		D2	D2	D2	D2	D2	D2	D2	D2			
C		D3	D3	D3	D3	D3	D3	D3	D3			
D		D4	D4	D4	D4	D4	D4	D4	D4			
E												
F												
G												
H												

Example of volumes for serial dilutions

Dilution	**Volume of thrombin pre-dilution (µl)**	**Buffer C (µl)**	**Nominal concentration (IU/ml)**
D1	200	0	0.5
D2	100 (D1)	100	0.25
D3	100 (D2)	100	0.125
D4	100 (D3)	100	0.0625

Using a multi-channel pipette, 40 µl of the Thrombin dilution range is transferred to the corresponding wells in the lower half of the plate (shown in yellow below).

Sample ID:		**S**	**A**	**B**	**C**	**C'**	**B'**	**A'**	**S'**			
	1	2	3	4	5	6	7	8	9	10	11	12
A		D1	D1	D1	D1	D1	D1	D1	D1			
B		D2	D2	D2	D2	D2	D2	D2	D2			
C		D3	D3	D3	D3	D3	D3	D3	D3			
D		D4	D4	D4	D4	D4	D4	D4	D4			
E		D1	D1	D1	D1	D1	D1	D1	D1			
F		D2	D2	D2	D2	D2	D2	D2	D2			
G		D3	D3	D3	D3	D3	D3	D3	D3			
H		D4	D4	D4	D4	D4	D4	D4	D4			

Clotting

The reaction is initiated by adding 60 µl of fibrinogen or plasma solution to the wells containing the 40 µl Thrombin solutions as quickly as possible (the final concentrations of thrombin in the reaction mixtures are 0.2, 0.1, 0.05, and 0.025 IU/ml). The solutions and the plate should be pre-warmed to 37 °C. The plate is read at 405 nm, 37 °C, for up to 2 hours, with readings every 30 seconds.

Performing assays with more than one substrate

If you would like to perform the assays with more than one substrate (plasma, fibrinogen, and/or chromogenic) it might be more convenient and time-effective to prepare a masterplate containing the thrombin dilutions, which can be transferred to a measurement plate or plates (for example, with fibrinogen in the top half and plasma or chromogenic substrate in the bottom half).

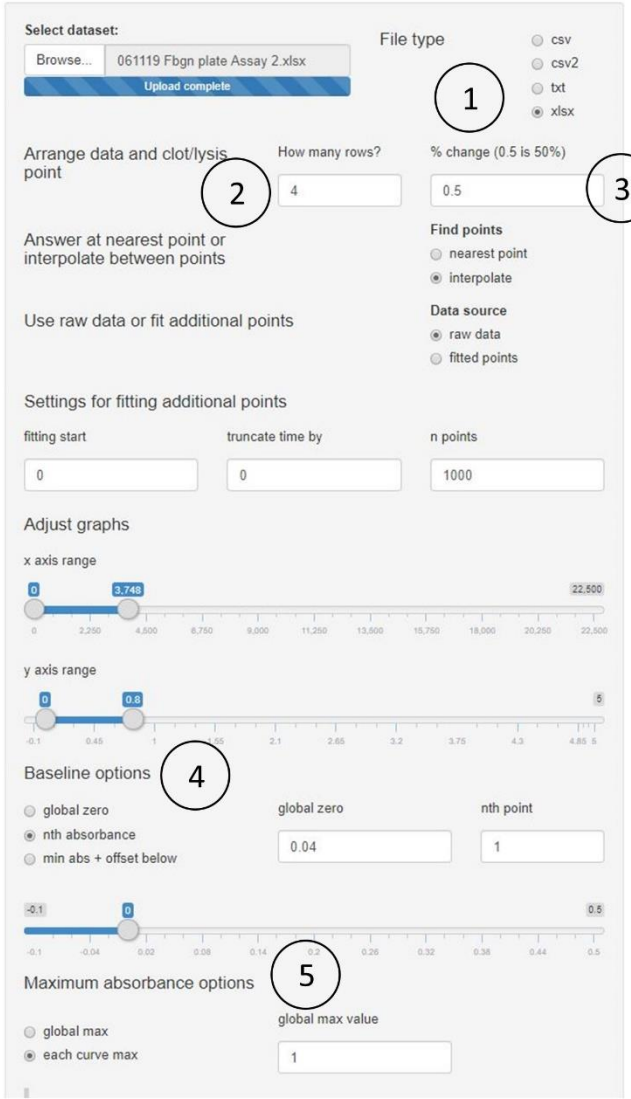
Data reporting

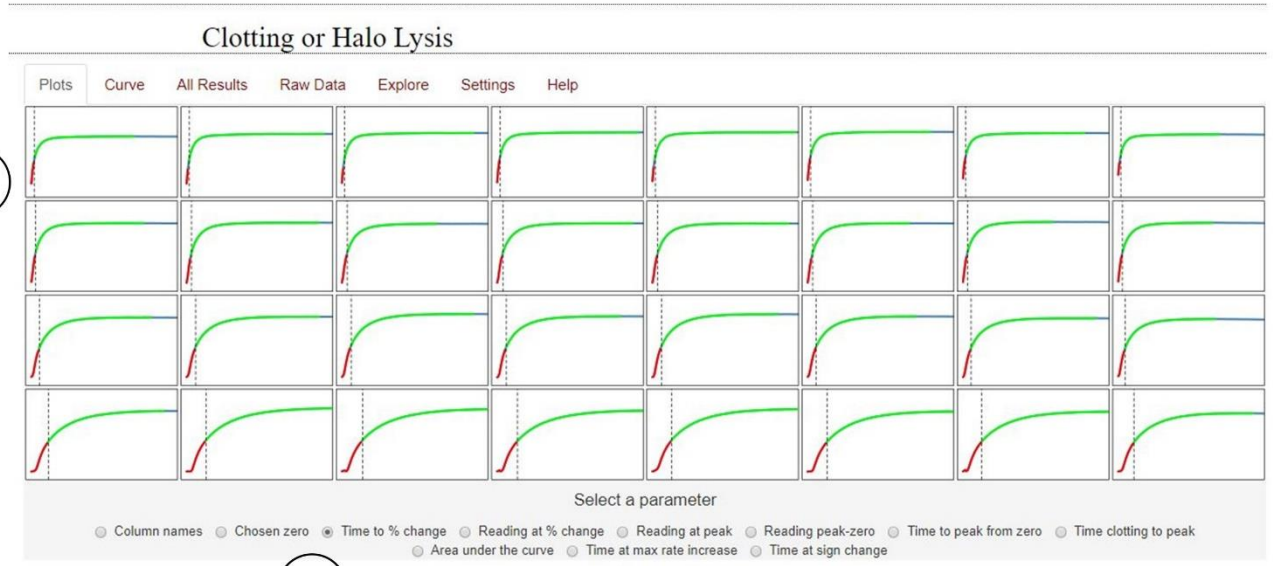
Clotting times are recorded as the time taken for half-maximal absorbance to be reached in all wells (including replicates and not just means).

A more time-effective way of calculating this for entire plates is to use the recently developed Shiny Apps. The following link can be used to analyse data, with instructions provided in the "Help" tab, https://drclongstaff.shinyapps.io/Clot_or_HaloCL or more detailed help can be found through links at https://drclongstaff.github.io/shiny-clots/. For further questions on using the app, please contact colin.longstaff@nibsc.org or matthew.locke@nibsc.org.

However, if you choose to use this method, we would still like to receive raw data as time versus absorbance files.

An example of the analysis is shown below:





all.change.Time for 50 % of maximum

1	2	3	4	5	6	7	8
75.66	72.76	73.70	75.25	74.94	77.48	76.70	67.86
113.20	129.56	140.28	151.12	142.63	131.43	122.98	113.53
220.57	239.66	247.19	246.67	246.57	229.41	216.38	188.84
459.04	522.19	551.10	542.93	510.47	522.69	500.54	416.90

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Notes:

1. Prepare file for upload (see notes on webpage) and specify format
2. Select number of rows in table
3. Select magnitude of parameter to be analysed (e.g. 50 % clotting)
4. Select baseline (1st point in this case)
5. Select maximum (maximum for each curve in this case)
6. Select the parameter to be analysed
7. 50 % clotting times are displayed in the table and colour-coded in graphical output

Example of chromogenic plate assay used at NIBSC

ASSAY PRINCIPLE

A synthetic chromogenic substrate (in this case S-2238) is incubated with a dilution range of thrombin in a microtitre plate and absorbance monitored over time in a plate reader. The amount of p-nitroaniline formed, and the absorbance of the solution at 405 nm, increases exponentially and is proportional to the thrombin concentration.

The aim of the study is to assign potencies to three candidate samples (labelled A, B, and C). The potencies of the test samples are determined by comparison with a reference preparation, in this case the WHO 2nd International Standard for Thrombin (code 01/580, Sample S), with an assigned potency of 110 IU per ampoule.

PREPARATION OF WORKING REAGENTS

Chromogenic substrate working solution

Substrate working solution is prepared by diluting stock substrate solution to 1 mM in Buffer B (e.g. 2 ml S-2238 stock (3 mM) is added to 4 ml Buffer B). Maintain working stock at 37 °C.

Thrombin dilutions

Each assay requires at least two replicate dilution ranges of 4 doses of thrombin from each ampoule S, A, B, and C. Dilutions are carried out in Buffer C (although Ca^{2+} is not required in the buffer). The most concentrated solution is 0.5 IU/ml and a range of doubling dilutions is prepared from this solution. Two pre-dilutions may be performed to make the 0.5 IU/ml solution as follows:

Sample	1st pre-dilution	2nd pre-dilution	Nominal concentration (IU/ml)
S	45 µl -> 1 ml	100 µl -> 1 ml	0.5 IU/ml
A	50 µl -> 1 ml	100 µl -> 1 ml	0.5 IU/ml
B	55 µl -> 1 ml	100 µl -> 1 ml	0.5 IU/ml
C	55 µl -> 1 ml	100 µl -> 1 ml	0.5 IU/ml

The 0.5 IU/ml dilution (designated D1) is then used to make three additional doubling dilutions (D2-D4) in Buffer C, using a multi-channel pipette in a 96-well plate (as shown in the example of a 96-well plate below, in blue).

S', A', B' and C' refer to replicate sets of dilutions (and pre-dilutions) made independently from the same ampoule.

96-well plate layout for serial dilutions

Sample ID:		**S**	**A**	**B**	**C**	**C'**	**B'**	**A'**	**S'**			
	1	2	3	4	5	6	7	8	9	10	11	12
A		D1	D1	D1	D1	D1	D1	D1	D1			
B		D2	D2	D2	D2	D2	D2	D2	D2			
C		D3	D3	D3	D3	D3	D3	D3	D3			
D		D4	D4	D4	D4	D4	D4	D4	D4			
E												
F												
G												
H												

Example of volumes for serial dilutions

Dilution	**Volume of thrombin pre-dilution (µl)**	**Buffer C (µl)**	**Nominal concentration (IU/ml)**
D1	200	0	0.5
D2	100 (D1)	100	0.25
D3	100 (D2)	100	0.125
D4	100 (D3)	100	0.0625

Using a multi-channel pipette, 40 µl of the Thrombin dilution range is transferred to the corresponding wells in the lower half of the plate (shown in yellow below).

Sample ID:		**S**	**A**	**B**	**C**	**C'**	**B'**	**A'**	**S'**			
	1	2	3	4	5	6	7	8	9	10	11	12
A		D1	D1	D1	D1	D1	D1	D1	D1			
B		D2	D2	D2	D2	D2	D2	D2	D2			
C		D3	D3	D3	D3	D3	D3	D3	D3			
D		D4	D4	D4	D4	D4	D4	D4	D4			
E		D1	D1	D1	D1	D1	D1	D1	D1			
F		D2	D2	D2	D2	D2	D2	D2	D2			
G		D3	D3	D3	D3	D3	D3	D3	D3			
H		D4	D4	D4	D4	D4	D4	D4	D4			

The reaction is initiated by addition of 60 µl the **substrate solution** to the wells containing the thrombin solutions using a multi-channel pipette (the final concentrations in the reaction mixtures are 0.2, 0.1, 0.05, and 0.025 IU/ml).

The plate is read kinetically at 37 °C for up to 2 hours (taking readings every 30 seconds) at a wavelength of 405 nm.

Data Reporting

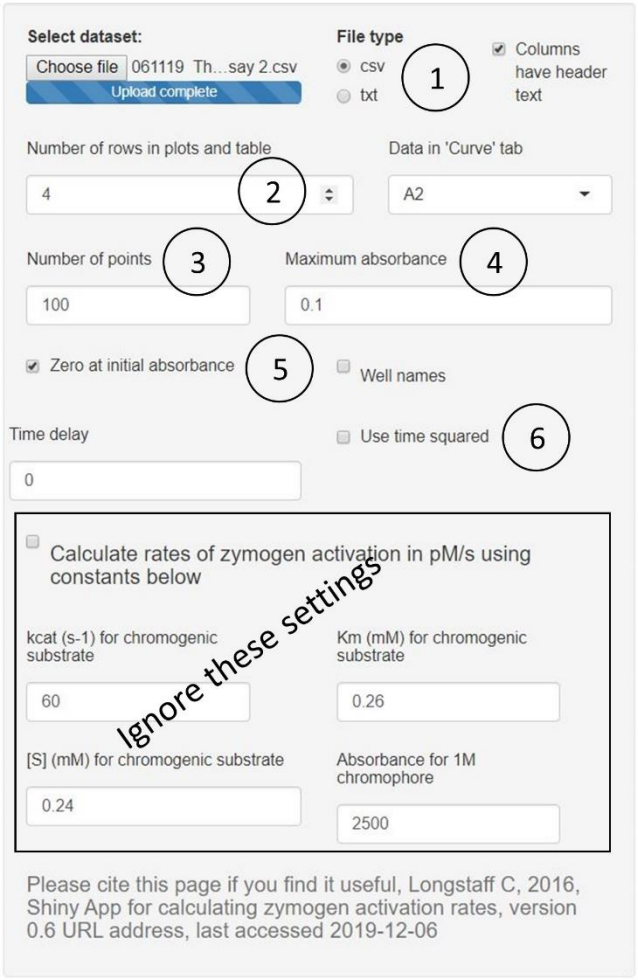
Rates can be described as the time taken to reach an OD of 0.1 (having adjusted the readings for the OD at time zero).

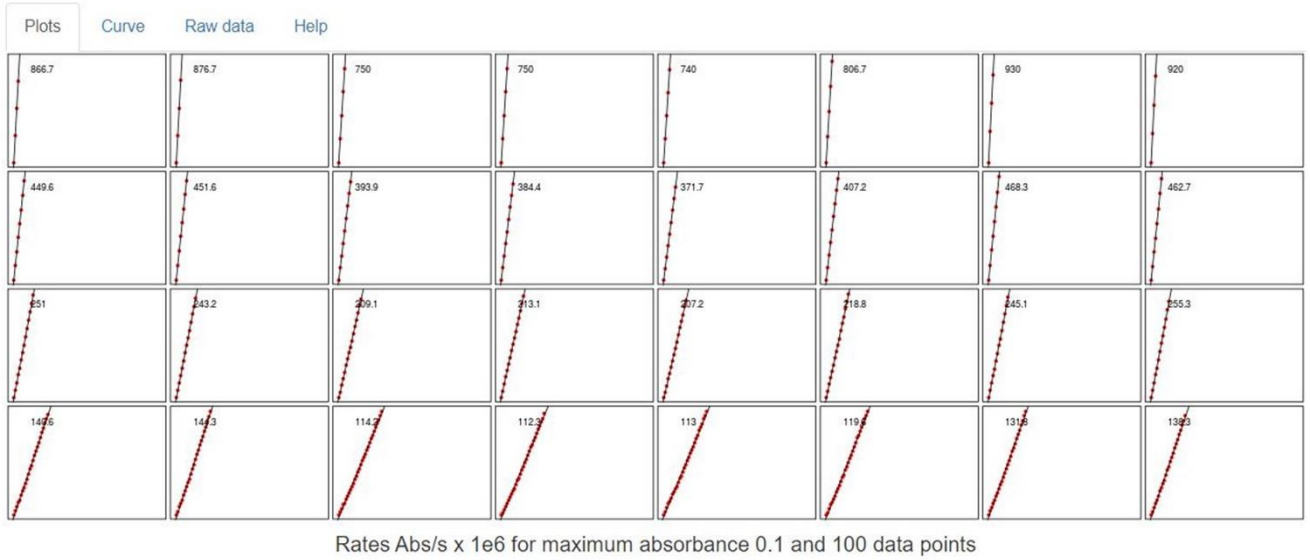
Alternatively, raw data can be exported as a .csv or .txt file for analysis using the Zymogen Activation Shiny App, https://drclongstaff.shinyapps.io/zymogenactnCL, and follow guidance in "Help" tab. More detailed help is available from links at https://drclongstaff.github.io/shiny-clots/.

Select maximum OD changes up to 0.1 arbitrary units to display rates in Abs/sec (in the example below, leave the "select for time squared" and "calculate rates in pM/s" boxes unchecked and ignore the other settings. However, if you choose to use this method, we still need to receive raw data as time versus absorbance files.

For further questions on using the App, please contact colin.longstaff@nibsc.org or matthew.locke@nibsc.org.

An example of the analysis is shown below:





	1	2	3	4	5	6	7	8
1	866.67	876.67	750.00	750.00	740.00	806.67	930.00	920.00
2	449.60	451.59	393.89	384.44	371.67	407.22	468.25	462.70
3	250.99	243.15	209.12	213.09	207.21	218.82	245.13	255.31
4	140.64	144.30	114.16	112.34	112.97	119.62	131.82	138.29

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Notes:

1. File must be in txt or csv format (see notes on webpage)
2. Specify number of rows in table
3. Select number of datapoints in your file (or to be analysed)
4. Select maximum absorbance change
5. Enable zero initial absorbance
6. Ensure Use time squared is deselected
7. Rates to specified absorbance change are shown in table

Example of automated coagulometer clotting assay used at NIBSC

ASSAY PRINCIPLE

For this method we adopted the 'Thrombin Time 2ml' (TT2) programme on the Werfen ACL TOP 550 analyser, although similar methods (such as Reptilase Time) on other analysers may be appropriate, or a method could adapted or programmed manually.

The TT2 method measures clotting with an acquisition time of 60 s and a delay of 3 s. The thrombin is mixed 1:1 with fibrinogen or plasma (80 µl of each, total volume 160 µl) in a cuvette and clotting is monitored by absorbance change over time. Results are reported as clotting times based on a threshold OD.

Assay modifications

We made two significant changes to the usual protocol for this method. Typically this method is used to test the clotting potential of human plasma. As such the reagent is thrombin and the samples are plasma. For our purpose the clotting enzyme is the test sample, and plasma (or purified fibrinogen) is the reagent. We therefore replaced thrombin as the reagent with plasma/fibrinogen, and the test was run with sample dilutions in the sample cups instead of plasma.

The second change was to use the 'extended time' version of the test (TT2E), which has an extended acquisition time of 300 s. Because we are using a dilution range, rather than the intended single point measurement, the higher sample dilutions produced clotting times outside of the normal 60 s. As a result the test 'fails' and an automated re-run of the assay is triggered using the extended protocol. This uses more sample and reagent and extends the time of the assay. We preferred to run the extended version TT2E by default.

PREPARATION OF WORKING REAGENTS

Fibrinogen stock solution

A 5 mg/ml fibrinogen **working stock** is prepared by adding 400 µl of 50 mg/mL fibrinogen stock solution to 3.6 ml buffer B. Maintain at room temperature.

Plasma working solution

If plasma is used instead of fibrinogen, a plasma **working stock** is prepared by diluting plasma 1:1 with buffer B. Maintain at room temperature.

Thrombin dilutions

Each assay requires at least two replicate dilution ranges of 4 doses of thrombin from each ampoule S, A, B, and C. All dilutions are carried out in Buffer C. The most concentrated solution is 4 IU/ml and a range of doubling dilutions is prepared from this solution.

Replicate pre-dilutions may be performed to make the 4 IU/ml solution as follows:

Sample	1st pre-dilution	Nominal concentration (IU/ml)
S	36 µl -> 1 ml	4 IU/ml
A	40 µl -> 1 ml	4 IU/ml
B	44 µl -> 1 ml	4 IU/ml
C	44 µl -> 1 ml	4 IU/ml

From the 4 IU/ml dilution (designated D1), make three additional doubling dilutions for each of S, A, B, and C in Buffer C. Maintain at room temperature.

Example of volumes for serial dilutions

Dilution	Volume of thrombin pre-dilution (µl)	Buffer C (µl)	Nominal concentration (IU/ml)
D1	400	0	4
D2	400 (D1)	400	2
D3	400 (D2)	400	1
D4	400 (D3)	400	0.5

Running the assay

1. Place 32x 2ml sample cups into the racks representing each of the 4 dilutions for each of the samples S, A, B and C and replicates S', A', B' and C'
2. Transfer at least 250 µl of each sample working dilution into the corresponding cup (remembering to follow the balanced design for each assay as described in the study protocol document)
3. Place the racks into the tracks on the sample area of the ACL TOP and lock into position
4. Enter a unique sample identification number in the ACL software e.g. Assay1 S D1
5. Select the required test (TT2E) and apply to all samples
6. Add the Fibrinogen or Plasma working solution to an appropriate sized reagent bottle
   NOTE: make sure the correct bottle size is selected for Thrombin in the material definition list, with an appropriate volume threshold (e.g. if using a 4 ml bottle change the volume threshold to 0.3 ml)
7. In the material definition list select the Thrombin reagent and de-deselect 'Enable lot management'. This will ensure no expiry date is registered for the material when it is loaded onto the machine
8. Place a blank label on the bottle to prevent the scanner registering the 'no reagent' barcode through the bottle
9. Place the reagent bottle containing fibrinogen (or plasma) into a reagent rack and load onto the machine. Select *Thrombin* as the identifier for this reagent
10. Place 2x 20 ml reagent bottles containing a 1/8 diluted Clean B solution (e.g. 2 ml Clean B + 14 ml water) into a dilution rack and a reagent rack and load onto the machine (e.g. bottle 1 in D1 and bottle 2 in any of R1-R4)
11. When all samples and reagents have been entered click on the 'perform' icon and the test run will begin
12. When the run is complete the results will print automatically. Please record the clotting times on the results sheets provided.

Calibration of the proposed WHO 3rd International Standard for Thrombin

CS656: Results sheet

Laboratory:

Name:

Method: *Please provide brief details e.g. equipment used, substrate – fibrinogen or plasma, chromogenic substrate, measurement parameters e.g. for clotting assays, time to O.D., % clotting, for chromogenic assays, indicate if rates or times are to a specific O.D.*

- **Please complete the results tables below, or provide the same information in a digital format (e.g. Excel). Please remember to include sample (pre-) dilution information.**
- **Please ensure that your results are presented as true raw data (e.g. clotting time) rather than as % or units relative to an in house standard.**
- **Please provide as much raw data as possible as csv, txt or Excel files.**

EXAMPLE

Nominal concentrations and 50% clotting times are in red as an example. Nominal concentration refers to the concentration of thrombin following all pre-dilution and dilution steps.

		RESULT e.g. time to 50% clotting in seconds							
	Nominal conc. (IU/ml)	**S**	**A**	**B**	**C**	**C'**	**B'**	**A'**	**S'**
D1	**0.5**	75.7	72.8	73.7	75.3	74.9	77.5	76.7	67.9
D2	**0.25**	113.2	129.6	140.3	151.1	142.6	131.4	123.0	113.5
D3	**0.125**	220.6	239.7	247.2	246.7	246.6	229.4	216.4	188.8
D4	**0.0625**	459.0	522.2	551.1	542.9	510.5	522.7	500.5	416.9

Pre-dilution information:

To obtain the 0.5 IU/ml solution (D1) above we perform two pre-dilution steps, an example of which is shown below in red.

Sample	1st pre-dilution	2nd pre-dilution	Nominal concentration (IU/ml) of D1
S	45 µl -> 1 ml	100 µl -> 1 ml	0.5
A	50 µl -> 1 ml	100 µl -> 1 ml	0.5
B	55 µl -> 1 ml	100 µl -> 1 ml	0.5
C	55 µl -> 1 ml	100 µl -> 1 ml	0.5

Assay 1

		RESULT							
	Nominal conc. (IU/ml)	**S**	**A**	**B**	**C**	**C'**	**B'**	**A'**	**S'**
D1									
D2									
D3									
D4									

Pre-dilution information:

Sample	**1st pre-dilution**	**2nd pre-dilution**	**Nominal concentration (IU/ml) of D1**
S			
A			
B			
C			

Assay 2

		RESULT							
	Nominal conc. (IU/ml)	**A**	**B**	**C**	**S**	**S'**	**C'**	**B'**	**A'**
D1									
D2									
D3									
D4									

Pre-dilution information:

Sample	1st pre-dilution	2nd pre-dilution	Nominal concentration (IU/ml) of D1
S			
A			
B			
C			

Assay 3

		RESULT							
	Nominal conc. (IU/ml)	**B**	**C**	**S**	**A**	**A'**	**S'**	**C'**	**B'**
D1									
D2									
D3									
D4									

Pre-dilution information:

Sample	1st pre-dilution	2nd pre-dilution	Nominal concentration (IU/ml) of D1
S			
A			
B			
C			

Assay 4

		RESULT							
	Nominal conc. (IU/ml)	**C**	**S**	**A**	**B**	**B'**	**A'**	**S'**	**C'**
D1									
D2									
D3									
D4									

Pre-dilution information:

Sample	1st pre-dilution	2nd pre-dilution	Nominal concentration (IU/ml) of D1
S			
A			
B			
C			

Appendix 2. List of participants. The order is not the same as the laboratory code.

Dr Andreas Hunfeld Jana Fötisch Paul-Ehrlich-Institut Paul-Ehrlich-Str. 51 – 59 Department of Hematology/Transfusion Medicine, Langen 63225, Germany	Serena Strobl Takeda/Baxter AG Lange Allee 24, Vienna, A-1220, Austria	Laure Cuignet, Adeline Gazan, Fatiha Rahmouni, Sabah Said, Wim Van Molle Sciensano, Quality of Vaccines and Blood Products Rue Juliette Wytsmanstraat 14, Brussels 1050, Belgium
Nuria Hosta Instituto Grifols Can Guasch, 2 08150 Parets del Vallès Barcelona, Spain	Prof Joost Meijers Kamran Bakhtiari Sanquin Research Dept. Molecular and Cellular Hemostasis Plesmanlaan 125 1066 CX Amsterdam The Netherlands	Minyoung Yoo GC Pharma R&D Centre 93, Ihyeon-ro 30beon-gil, Giheung-gu, Yongin-si, Gyeonggi-do, Republic of Korea
Dr Moti Alberstein Omrix Biopharmaceuticals Ltd. MDA-Blood Bank Tel-Hashomer Hospital POB 888, KIRYAT-ONO 5510801, Israel	Prof Robert Ariëns, Helen McPherson University of Leeds Discovery and Translational Science Department, Leeds Institute of Cardiovascular And Metabolic Medicine The LIGHT Laboratories Clarendon Way, Leeds LS2 9NL, UK	Dr Mikhail Ovanesov, Stepan Surov, Leonid Parunov, Hemostasis Branch Division of Plasma Protein Therapeutics FDA-CBER 10903 New Hampshire Ave, Silver Spring, MD 20993, USA
Dr Patrizia Caprari Dr Maria Teresa Pasquino Dr Luisella Luchetti. National Centre for the Control and Evaluation of Medicines, Istituto Superiore di Sanità Viale Regina Elena, 299 00161, Rome, Italy	Shakira Govind Lu Liu Therapeutic Goods Administration, Department of Health, PO Box 100, Woden ACT, Canberra 2606, Australia	Dr Nikolaus Binder Technoclone Brunner Str. 67, Vienna 1230 Austria
Daniel Polasek, Renate Kaiser, Sylke Huber, Patrick Wenz Octapharma Pharmazeutika Produktionsges.m.b.H Oberlaaer Straße 235,Vienna, 1100, Austria	Dr Christoph Kefeder The Federal Office for Safety in Health Care (BASG) Department for Analysis of Biological Medicinal Products Possingergasse 38, Vienna 1160, Austria	Prof Joost Meijers Wil Kopatz Amsterdam UMC Dept. Experimental Vascular Medicine Meibergdreef 9 1105 AZ Amsterdam The Netherlands

Dr. Jai Prakash Prasad, Dr Meena Kumari Mrs. Y. Madhu Dr. Varun Singh National Institute of Biologicals Ministry of Health & Family Welfare, Government of India A-32, Sector – 62, NOIDA – 201 309, U. P., India	Sang-Mi Park National Institute of Food and NIFDS (National Institute of Food and Drug Safety Evaluation), Blood Products Division, 187 Osongsaengmyeng 2-Ro, Osong-eup, Heungdeok-gu, Cheongju-si 28159 Republic of KOREA	Dr Matthew Locke Haemostasis Section, Biotherapeutics Division, National Institute for Biological Standards and Control (NIBSC) South Mimms, UK
Prof Anetta Undas, Dr Michal Zabczyk Jagiellonian University Medical College, Kraków, Poland		

Appendix 3. Participants' Response Sheet

Medicines & Healthcare products Regulatory Agency

NIBSC Confidence in Biological Medicines

An international collaborative study to establish the WHO 3rd International Standard for Thrombin

Participants' Response Sheet

After considering the report of the collaborative study, we now ask for your opinion and any feedback on the proposal made in the report.

Proposal
Preparation 19/188 is proposed as the WHO 3rd International Standard for Thrombin with a potency of 90 IU per ampoule.

Lab name and number: …….

☐	☐
I agree with the proposal	**I do not agree with the proposal**

If you have any further comments, we will be happy to receive them in the space below, and they will be incorporated into subsequent reports.

As a thank you for taking part in the study we would like to offer you 6 free ampoules of 19/188. Please respond below if you would like to take up this offer.

☐ Yes, I would like 6 ampoules of 19/188

☐ No, I would not like 6 ampoules of 19/188

Please respond by email to Matthew Locke at matthew.locke@nibsc.org

National Institute for Biological Standards and Control
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World Health Organization

Appendix 4. Draft Instructions for Use for 19/188

Medicines & Healthcare products Regulatory Agency

NIBSC Confidence in Biological Medicines

WHO International Standard
WHO 3rd International Standard for Thrombin
NIBSC code: 19/188
Instructions for use
(Version 1.00, Dated)

1. INTENDED USE

The above named Standard has been developed to replace the 2nd International Standard for Thrombin (01/580). The potency of the new Standard is 90 IU/ampoule.

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. 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 potency was assigned to this Standard through an international collaborative study comprising 20 laboratories in 13 countries. Participants were requested to perform their in-house methods for thrombin potency determinations. These were performed with fibrinogen or plasma substrates or chromogenic methods. The potency of the candidate materials was calculated relative to the 2nd International Standard for Thrombin (01/580) using parallel line analysis. The conclusion of the study was candidate material 19/188 was a suitable replacement standard for 01/580 with a potency of 90 IU/ampoule. This was based on results from fibrinogen and plasma clotting assays. Results from chromogenic assays were not used in the calculation of this value.

4. CONTENTS

Country of origin of biological material: United Kingdom.
The thrombin used to make the Standard was generously provided by a manufacturer as a purified solution of human alpha-thrombin prepared from pooled plasma. The material was shipped to NIBSC as a frozen solution where it was then thawed and diluted to a concentration of approximately 90 IU/ml in a solution of 10 mM Hepes, pH 7.4, containing 0.15M NaCl and 5 mg/ml human albumin solution.

5 mL DIN ampoules were filled with 1 mL aliquots of the diluted material and lyophilised following NIBSC procedures. A total of 9720 ampoules of 19/188 were available for use. Precision of the fill was monitored by check-weights evenly spaced throughout the total fill. The results are expressed as the % coefficient of variation (cv), where n is the number of ampoules sampled to determine each parameter: mean filling weight = 1.0070 g (cv = 0.14 %, n = 343); mean dry weight = 0.01643 g (cv = 1.16 %, n = 6); mean residual moisture content = 0.49 % (cv = 27.68 %, n = 12); mean oxygen headspace = 0.43 % (cv = 33.92 %, n = 12).

The alpha thrombin content of the Thrombin Standard is not known exactly but the international collaborative study demonstrated by the ratio of clotting to chromogenic activity that it is very similar to the previous 2nd International Standard for Thrombin (01/580) (1), which in turn was very similar to the 1st International Standard for Alpha Thrombin (89/588) (2).

5. STORAGE

Unopened ampoules should be stored in the dark at or below -20ºC.
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. Various types of ampoule breaker are available commercially. To open the ampoule, tap the ampoule gently to collect material at the bottom (labelled) end and follow manufactures instructions provided with the ampoule breaker.

7. USE OF MATERIAL

No attempt should be made to weigh out any portion of the freeze-dried material prior to reconstitution
To reconstitute, allow the ampoule to warm to room temperature and ensure that the lyophilised material is all in the base of the ampoule before carefully snapping off the top of the ampoule. The contents should be reconstituted using 1 mL of distilled water and mixed gently to produce a clear, colourless solution. This solution should be stored on ice and used as soon as possible by dilution into appropriate assay buffer under conditions defined for your assay. Following reconstitution, the activity is stable for several hours when the solution is maintained on ice. However, the potency is not guaranteed after further freezing and thawing of the reconstituted solution.

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. It is the policy of WHO not to assign an expiry date to their international reference materials and they remain valid with the assigned potency until withdrawn or amended. Predictions on long term stability are made by monitoring ampoules stored under accelerated degradation conditions over time.

9. REFERENCES

(1) Whitton C, Sands D, Lee T, Chang A, Longstaff C. A reunification of the US ("NIH") and International Unit into a single standard for Thrombin. Thromb Haemost. 2005;93(2):261-6.
(2) Gaffney PJ, Heath AB, Fenton JW, 2nd. A collaborative study to establish an international standard for alpha-thrombin. Thromb Haemost. 1992;67(4):424-7.

A report of the collaborative study to calibrate the standard is available from WHO, reference WHO/BS/XXXXXX

10. ACKNOWLEDGEMENTS

We are grateful to all the participants that took part in the collaborative study, and to the FXIII and Fibrinogen Subcommittee of the Scientific and Standardization Committee (SSC) of the International Society on Thrombosis and Haemostasis (ISTH).

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

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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

Physical 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: 10 mg
Toxicity Statement: Toxicity not assessed
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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