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An international collaborative study to establish the WHO 4th International Standard for Streptokinase (16/358)

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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 **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

Stocks of the existing WHO 3rd International Standard (IS) for Streptokinase are low and a replacement is required. Two candidate replacements were donated by manufacturers and formulated, filled and freeze-dried into sealed glass ampoules (coded 16/356 (sample B) and 16/358 (sample C)). An international collaborative study was organised to assign potency values to the candidate standards relative to the current IS (00/464, sample S). A fourth sample (88/824, sample A) used in the previous two international collaborative studies to establish the 2nd and 3rd IS, was also included.

A total of 15 laboratories from 9 countries were recruited to take part in the study, all of which returned results, using chromogenic and/or fibrinolytic methods. Assays from each laboratory were used to calculate laboratory geometric mean potencies, which were combined to give overall geometric mean potencies for each method. There was very good agreement between the methods, which were combined to give overall potencies of 909.1 IU/ampoule for 16/356 and 1012.7 IU/ampoule for 16/358, from a total of 69 independent assays. Inter-laboratory variability, expressed as the geometric coefficient of variation (GCV), was 8.2 % and 7.2 % for 16/356 and 16/358, respectively. The potency obtained for 88/824 (461 IU/ampoule, GCV = 11.3 %) was the same as the result obtained in 2000 to establish the 3rd IS, suggesting excellent long-term stability of the material and good continuity of the unit. Accelerated degradation studies on 16/356 and 16/358 indicate the candidate standards are very stable, in agreement with results for previous streptokinase standards.

Both 16/356 and 16/358 are suitable replacements for the 3rd IS. Based on slightly lower inter-laboratory variation, and better agreement between assay methods, it is proposed that preparation 16/358 is established as the WHO 4th IS for streptokinase, with a potency of 1013 IU per ampoule.

INTRODUCTION

Streptokinase (SK) was introduced as a therapy for Acute Myocardial Infarction (AMI) over 50 years ago, and is still used in developing countries as a cheap and effective treatment [1]. Its worldwide importance as a thrombolytic is highlighted by its inclusion as a WHO essential medicine [2]. As for all thrombolytics, the narrow therapeutic window for SK requires balancing effective thrombolysis without increasing risk of major bleeding. The current 3rd WHO International Standard (IS) for SK (00/464) is distributed to all parts of the world to calibrate therapeutic SK products, ensuring accurate potency labelling and dosing.

The use of SK as a thrombolytic stems from its ability to bind and activate plasminogen to active plasmin. Unlike other plasminogen activators, SK does not possess proteolytic activity, and is not a true enzyme. It forms a 1:1 stoichiometric complex with plasminogen or plasmin and activates the zymogen through a non-proteolytic intramolecular cleavage. The resulting

complex can proteolytically activate free plasminogen to plasmin, which can subsequently dissolve the thrombus.
SK potency assays measure the ability of a SK preparation to generate plasmin from plasminogen and may be divided into solution assays (chromogenic and fluorogenic) and fibrin-based assays (clot lysis). Fibrin-based assays take a variety of formats, including tube-based bubble release assays (time taken for the release of trapped bubbles at the point of clot lysis) and ball-drop assays (measuring the time taken for a steel or glass ball to pass through a clot). Turbidity measurements in microtitre plates offer a convenient method for determining lysis times with larger sample sizes, particularly with recent improvements in standardization of data analysis [3]. Both solution- and fibrin-based methods have been validated in international collaborative studies and gave equivalent results when using the 2nd and 3rd IS for SK [4, 5]. The British and European Pharmacopoeias both adopted the solution chromogenic method for SK potency in 2004, replacing a euglobulin fibrin clot lysis method [6].

All commercial SK products are derived from the H46A isolate of group C *Streptococcus equisimilis*, where it functions to enhance fibrinolysis and destruction of extracellular matrix proteins to promote dissemination and virulence. The majority of products are native proteins obtained from filtrates of group C bacterial cultures, although some are produced recombinantly in *Escherichia coli* as a generic alternative. These recombinant versions have been shown to vary in activity depending on the presence or absence of fibrin, when compared to the native product [7, 8].

The 1st International Standard (IS) for SK was established in 1964 (coded 62/7), following trials confirming the effectiveness of SK as a thrombolytic [9]. This low purity preparation also included potency assignment for contaminating streptodornase. Subsequent 2nd (88/826) and 3rd (00/464) IS for SK were filled from high purity SK preparations to reflect preparations used clinically [4, 9].

Stocks of the 3rd IS for SK are now running low, and a replacement is required. This report describes the preparation of two candidate samples and the collaborative study to assign their potencies and establish the 4th IS for SK.

MATERIALS

Two manufacturers kindly donated samples of therapeutic streptokinase, which were native products derived from group C culture filtrates. These licensed products were Streptase donated by CSL Behring (lot # F5744411) and Biofactor Streptokinase donated by Lyocontract (lot # S160809). The formulation, dilution and freeze-drying were based on previous formulations for the 3rd IS for SK [5]. Briefly, material was reconstituted and diluted in 10 mM HEPES pH 7.4, 150 mM NaCl, 5 mg/ml human albumin (Zenalb-20, BPL, UK) to a final concentration of approximately 1000 IU/ml. Human albumin had been previously batch-release tested and was negative for viral and proteolytic contamination. 5 ml DIN ampoules were filled with 1 ml aliquots of the diluted material, lyophilised following NIBSC procedures and the ampoules stored at NIBSC (Potters Bar, UK) at -20 °C. The candidates were coded 16/356 (sample B) and 16/358 (sample C). Detailed characteristics of the ampouled materials are given in Table 1, which conform to WHO guidelines and recommendations.

DESIGN OF THE STUDY

The study consisted of four samples, one of which was the 3rd IS for SK (designated sample S, 00/464), one of which was a preparation used in the studies to establish the 2nd and 3rd IS (designated sample A, 88/824), and the two candidate preparations (samples B and C, coded 16/356 and 16/358, respectively).

Participants were asked to perform 4 independent fibrin clot lysis (fibrinolytic) or chromogenic assays to compare the potency of samples A, B, and C, relative to sample S, using fresh ampoules for each assay. An example clot lysis protocol was provided, based on clotting purified fibrinogen with thrombin in the presence of plasminogen and a range of SK, in a microtitre plate format. A method for the chromogenic assay was also provided, based on monitoring rates of plasminogen activation in solution over a dose range of SK. Suggestions for randomisation and dilution regimes were included, following prior fitness-for-purpose testing at NIBSC. Participants were also free to use their own methods. The study protocol and assay methods are included in Appendix 1.

A results sheet was provided where participants were asked to record experimental details and record clot lysis times (described as time to 50% lysis, or half maximal absorbance) or rates of plasmin formation (for absorbance changes up to 0.1 absorbance units). Whilst detailed analysis was not required, participants could perform and send in their own calculations. To help with this links were provided to online Apps, written in the programming language R, to analyse data and obtain clot lysis times or reaction rates [3]. Participants were also asked to return raw data, 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.

Data from each assay was used to calculate the relative potency of samples A, B, and C, against sample S, by parallel line analysis using the software program CombiStats [10]. Tests of validity (significance of non-linearity and non-parallelism) were performed at the 1% level ($p<0.01$). Any deviations from linearity or parallelism were investigated further, with non-linearity 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). Assays with correlation coefficients below 0.95 were excluded, as they indicate variance from the model. Non-parallelism was further assessed by comparing the ratios of fitted slopes for the standard relative to the samples. A ratio between 0.90 – 1.11 was considered acceptable.

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. As there was good agreement between methods, an overall mean potency was calculated, combining assays from all methods. 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) [11].

PARTICIPANTS

A total of 15 laboratories participated in the study, all of which returned results. The participating laboratories were from a wide geographical area, including Germany (2), India (3), UK (3), Australia (2), Japan, Hungary, Canada, Netherlands, and France. Of these, 2 were regulatory, 5 were industrial, and 8 were academic. Laboratories were assigned a code and the list of participants is provided in Appendix 2.

RESULTS

Assay data summary

Of the 15 participating laboratories, 9 performed clot lysis (fibrinolytic) experiments, contributing 31 assays (after exclusions, described below). Two of the laboratories (1 and 5) performed endpoint assays using plasma, with lysis times described as the time taken for a ball placed on top of a lysing clot to sink to the bottom of the tube. The other 6 laboratories performed clot lysis with fibrinogen in a microtitre plate, measuring clot turbidity over time. Eleven laboratories performed chromogenic measurements (contributing 38 assays after exclusions), two of which were endpoint, with the rest measuring plasminogen activation over time (kinetic).

Assay data analysis and deviations

Laboratories 1 and 5 used three SK doses instead of four for the plasma clot lysis assays, with a single dilution series measured in triplicate (laboratory 1) or duplicate (laboratory 5). Laboratories 3 and 5 used chromogenic endpoint assays to determine SK potency. Laboratory 3 followed the European Pharmacopoeia (EP) method for SK potency determination, with two independent dilution series comprising three dilutions measured. Assay 4 was removed from the analysis due to significant non-parallelism. Laboratory 5 measured a single dilution series comprising three dilutions, each in quadruplicate.

Laboratory 6 followed an in-house protocol for clot lysis, using SK dilutions suggested in the NIBSC protocol. Assay 1 was removed due to a low correlation coefficient. Assays 3 and 4 each had a dilution series which failed to give a dose-response, with the remaining dilution series giving significant non-linearity and non-parallelism. Based on this, assays 3 and 4 were also removed from the analysis.

Clot lysis assay 2 from laboratory 11 was removed due to lack of dose response, and the highest dose in assay 3 from laboratory 12 was removed due to non-linearity.
Laboratory 13 performed 3 assays for both chromogenic and clot lysis methods, instead of 4.
Laboratory 14 performed two clot lysis and two chromogenic assays. However, 3 of the 4 assays failed to give a dose-response, and only a single chromogenic assay was included in the analysis.

Summary of results

Detailed values of the individual laboratory mean potencies of samples A, B, and C, relative to sample S, from chromogenic and clot lysis assays are listed in Tables 2 and 3, respectively. There was good agreement between the two assay methods, which differed by only 2.2, 2.8, and 1.6% for samples A, B, and C, respectively. These differences were not statistically significant (unpaired, two-sided t-test, assuming equal variances, with 5% significance level),

allowing overall mean potencies for each sample to be calculated by combining potencies for all assay methods, summarised in Table 4.

Overall potency estimates were 460.8, 909.1, and 1012.7 IU/ampoule for samples A, B, and C, respectively, relative to sample S (1030 IU/ampoule), from a total of 69 assays. The value for sample C is consistent with the expected potency based on the activity of the bulk material used for the fill (~ 1000 IU/ml), whereas sample B is slightly lower. The previous study to establish the 3rd IS for SK obtained a potency of 461 IU/ampoule for sample A, the same as the estimate in this study.

Variability between the laboratories, expressed as the inter-laboratory % GCV, was low, being less than 10% for samples B and C for each method and in the overall analysis. The inter-laboratory GCV for sample A was slightly higher at 11.3%.

Variability within each laboratory, expressed as the intra-laboratory % GCV, ranged from 0.7 to 28.4 for sample A, 0.8 to 30.4 for sample B, and 1.3 to 33.3 for sample C. While the range appears quite wide, around half of the laboratories had GCVs below 10%, similar to previous SK studies.

Summary results are also presented in graphical form as a histogram of laboratory geometric means, colour coded by method, in Figure 1. The histograms illustrate good agreement between laboratories for samples A, B, and C, and good agreement between chromogenic and fibrin-based methods. This is important, since some SK molecules with variations in protein sequence or incomplete N-terminal processing (such as recombinant SK), have dramatically different potencies in the presence or absence of fibrin [8].

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 (-70 °C, -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 [12].

Potency estimates were obtained for samples B and C following 18 months storage at elevated temperatures, relative to samples stored at -20 °C, using an in-house chromogenic method (protocol in Appendix 1). The results, shown in Table 5, provided a good fit to the Arrhenius model and predicted potency losses of 0.018 % and 0.006 % per year at -20 °C, for samples B and C, respectively. This indicates the candidate standards are very stable, as expected from similar studies on previous SK standards [4, 5].

Prior to the current study, potency estimates were calculated for ampoules of the 3rd IS (00/464), which had been stored for 17 years at elevated temperatures. The results, using chromogenic and fibrinolytic methods, show that 00/464 is extremely stable, with only minor losses in activity per year (Table 6).

A number of ampoules of the candidate 4th IS (16/356 and 16/358) remain in storage at elevated temperatures for ongoing stability monitoring with chromogenic and fibrinolytic methods.

Bench stability following reconstitution

We investigated the stability of samples B and C following reconstitution, to replicate conditions experienced during a routine assay period. Reconstituted material was transferred to stoppered tubes and placed on melting ice for 4, 8 or 24 hours, and activity assayed against a freshly reconstituted ampoule using an in-house chromogenic assay (Appendix 1). The results in Table 7 show that the reconstituted samples B and C are very stable after reconstitution, with little loss in activity over the assay period.

Consistency between studies

Sample A (88/824) has been included in three international collaborative studies and potencies calculated against the 1st (62/7), 2nd (88/826), 3rd (00/464), and sample C (16/358) are shown in Table 8. In the current study, the potency of sample A was 461 IU/ampoule versus sample S (3rd IS), the same as the result obtained in 2000 to establish the 3rd IS. When calibrated against sample C (16/358, 1013 IU/ampoule), the potency is 460 IU/ampoule (detailed values are listed in Table 9). Thus, 88/824 has shown a variability of only 1.9% over four International Standards spanning 30 years. This illustrates excellent stability of the SK standard under our formulation and freeze-drying conditions, and good continuity of the unit and assay methods.

Proposal

Both samples B and C are suitable replacements for the 3rd IS for SK. It is proposed that sample C (16/358) is adopted as the WHO 4th International Standard for SK, with a potency of 1013 IU per ampoule (rounded from 1012.7 IU per ampoule). This is based on better agreement in potency estimates between assay methods, and lower inter-laboratory variation, compared to sample B (16/356).

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 16/358 as the 4th IS for SK with a potency of 1013 IU per ampoule. There were no additional comments to be addressed.

EXPERT REVIEW BY THE SSC/ISTH

The study report was distributed to experts of the SSC/ISTH by Prof. Joost Meijers, SSC/ISTH-WHO Liaison Officer, for further review. Responses were received by 8 experts and all agreed with the proposal. Two comments were made by two different reviewers:

Comment 1: "Very minor point: Fig. 1 has very small font size that is hard to read."

The font size in Figure 1 has been increased to make it clearer.

Comment 2: “Minor comment: Even though I fully agree with the proposal to establish 16/358 as the WHO 4th IS for streptokinase, I was wondering, since the differences (interlaboratory variation, …) with 16/356 are probably not significant, if 16/356 could also somehow be recovered as an IS for streptokinase (since all the work has been done anyway and according to the data this preparation also fulfils all criteria). Probably a matter of internal procedures, but I still wanted to raise this point.”.

This is an important point. At NIBSC we have an ongoing program to monitor the stability of our IS using accelerated degradation. With this in mind, 16/356 could be used as an alternative IS should there be unexpected stability issues with 16/358 in the future (although given the excellent stability of the SK IS, this may seem unlikely). It is also possible that 16/356 could replace 16/358 when stocks are exhausted. We are also open to the possibility of distributing 16/356 as a secondary working standard should the need arise, and we will advise high-volume users of the IS of this possibility.

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The Fibrinolysis SSC Subcommittee of the ISTH.

The team from the Centre for Biological Reference Materials at NIBSC for filling the samples and organizing shipping to the study participants.

Table 1. Characteristics of the filled batches of ampoules for candidate International Standards 16/356 and 16/358. Precision of the fill was monitored by check-weights evenly spaced throughout the total fill. The results are expressed as the % coefficient of variation, where n is the number of ampoules sampled to determine each parameter.

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.

	Sample B (16/356)	**Sample C (16/358)**
Date of fill	23/02/2017	02/03/2017
Number of ampoules	9715	7149
Mean fill mass g (CV)	1.0078 (0.12%), n=339	1.0080 (0.17%), n=256
Mean dry weight g (CV)	0.0164 (0.24%), n=6	0.0113 (5.46%), n=6
Mean residual moisture % (CV)	0.3440 (12.32%), n=12	0.6278 (28.81%), n=12
Mean oxygen head space % (CV)	0.23 (31.28%), n=12	0.24 (55.51%), n=12

Table 2. Potency estimates (IU per ampoule) of samples A, B, and C, relative to sample S, the 3rd IS for SK, in chromogenic assays.

		Sample A (88/824)			Sample B (16/356)			Sample C (16/358)		
Laboratory code	**No. of assays**	**Potency (IU/ampoule)**	**95% CI**	**Intra-lab GCV %**	**Potency (IU/ampoule)**	**95% CI**	**Intra-lab GCV %**	**Potency (IU/ampoule)**	**95% CI**	**Intra-lab GCV %**
2	4	**454.10**	421.37-489.36	**4.8**	**864.30**	828.00-902.19	**2.7**	**967.90**	923.56-1014.69	**3.0**
3	3	**440.77**	408.19-479.95	**6.3**	**935.70**	854.96-1029.14	**7.4**	**922.54**	782.65-1077.71	**13.0**
4	4	**473.26**	368.65-607.54	**17.0**	**1075.84**	950.45-1217.76	**8.1**	**1052.51**	982.26-1127.79	**4.4**
5	4	**462.43**	453.79-471.24	**1.2**	**975.96**	943.64-1009.39	**2.1**	**978.97**	956.98-1001.46	**1.4**
7	4	**568.35**	382.58-844.33	**28.2**	**915.62**	689.71-1215.53	**19.5**	**1124.60**	791.64-1597.60	**24.7**
8	4	**474.90**	448.95-502.35	**3.6**	**962.70**	790.18-1172.87	**13.2**	**1106.37**	1003.69-1219.55	**6.3**
11	3	**468.30**	396.75-552.75	**11.0**	**919.84**	768.45-1101.06	**12.0**	**1020.71**	898.31-1159.79	**8.4**
12	4	**351.87**	253.05-489.28	**23.0**	**760.34**	498.65-1159.34	**30.4**	**893.96**	566.14-1411.63	**33.3**
13	3	**440.88**	375.06-518.26	**6.7**	**874.27**	583.81-1309.26	**17.7**	**976.91**	600.99-1587.99	**21.6**
14	1	**460.94**	360.32-588.27	**-**	**926.04**	723.58-1182.00	**-**	**1104.12**	864.77-1412.16	**-**
15	4	**451.41**	388.92-523.94	**9.8**	**948.98**	769.87-1169.77	**14.0**	**1105.30**	920.95-1326.55	**12.2**
Total	**38**									
	Geometric mean	**456.34**	**Inter-lab GCV %**	**11.7**	**920.53**	**Inter-lab GCV %**	**9.0**	**1020.14**	**Inter-lab GCV %**	**8.3**

Table 3. Potency estimates (IU per ampoule) of samples A, B, and C, relative to sample S, the 3rd IS for SK, in clot lysis (fibrinolytic) assays.

		Sample A (88/824)			Sample B (16/356)			Sample C (16/358)		
Laboratory code	**No. of assays**	**Potency (IU/ampoule)**	**95% CI**	**Intra-lab GCV %**	**Potency (IU/ampoule)**	**95% CI**	**Intra-lab GCV %**	**Potency (IU/ampoule)**	**95% CI**	**Intra-lab GCV %**
1	4	**481.27**	458.44-505.24	**3.1**	**883.82**	798.77-977.93	**6.6**	**1081.71**	1042.53-1120.28	**2.3**
5	4	**480.56**	475.52-485.65	**0.7**	**985.63**	973.97-997.42	**0.8**	**983.83**	963.65-1004.44	**1.3**
6	1	**516.78**	423.78-631.20	**-**	**981.81**	804.52-1197.83	**-**	**1074.33**	880.89-1311.82	**-**
7	4	**520.37**	509.30-531.69	**1.4**	**962.27**	824.05-1123.67	**10.2**	**993.13**	782.97-1259.70	**16.1**
8	4	**374.39**	284.77-492.22	**18.8**	**896.67**	773.98-108.80	**9.7**	**939.29**	807.78-1092.22	**9.9**
9	4	**470.57**	405.72-545.78	**9.8**	**846.11**	732.12-977.86	**9.5**	**1010.30**	931.68-1095.56	**5.2**
10	4	**489.70**	329.07-728.72	**28.4**	**809.86**	541.06-1212.19	**28.8**	**971.61**	845.75-1116.2	**9.1**
11	3	**478.14**	349.08-654.92	**13.5**	**853.41**	698.7-1042.38	**8.4**	**1072.35**	709.57-1620.61	**18.1**
13	3	**406.93**	317.13-512.15	**10.6**	**855.69**	488.69-1438.91	**25.3**	**921.25**	593.40-1430.25	**19.4**
Total	**31**									
	Geometric mean	**466.39**	**Inter-lab GCV %**	**11.5**	**895.22**	**Inter-lab GCV %**	**7.4**	**1003.75**	**Inter-lab GCV %**	**6.1**

Table 4. Summary of data analysis for potency determination of samples A, B, and C, relative to sample S, the 3rd IS for SK.

Assay Method	No. of assays	Mean Potency (IU/ampoule)					
		Sample A (88/824)	Inter-lab GCV (%)	Sample B (16/356)	Inter-lab GCV (%)	Sample C (16/358)	Inter-lab GCV (%)
Clot Lysis	31	466.4	11.5	895.2	7.4	1003.8	6.1
Chromogenic	38	456.3	11.7	920.5	9.0	1020.1	8.3
Overall	**69**	**460.8**	**11.3**	**909.1**	**8.2**	**1012.7**	**7.2**

Table 5. Potency remaining for candidate IS after 18 months storage at the indicated temperature, relative to ampoules stored at -20 °C. Each result is based on a combined potency from two ampoules assayed separately in duplicate. Assays were performed with the chromogenic method described in the study protocol (found in Appendix 1).

	% potency remaining relative to -20 °C			
	Sample B (16/356)		Sample C (16/358)	
Storage temperature (°C)	Mean	95% CI	Mean	95% CI
+4	99.3	95.1-103.7	98.5	92.3-105.2
+20	93.8	89.8-98.0	96.4	90.3-103.0
+37	92.3	84.7-100.6	94.2	88.3-100.6
+45	85.9	76.0-97.2	87.3	77.8-97.9
+56	68.2	60.6-76.8	69.4	65.0-74.2
Predicted loss per year (%)	**0.018**		**0.006**	
Upper 95% CI of potency loss (%)	**0.205**		**0.076**	

Table 6. Percent potency remaining for the 3rd IS for SK (00/464) after 17 years storage at the indicated temperature, relative to ampoules stored at -20 °C. Each result is based on a combined potency from two ampoules assayed separately in duplicate. Assays were performed with the chromogenic or fibrinolytic methods described in the study protocol (found in Appendix 1).

	% potency remaining relative to -20 °C			
	Clot lysis		Chromogenic	
Storage temperature (°C)	Mean	95% CI	Mean	95% CI
+4	97.1	90.9-103.6	99.8	90.3-110.3
+20	92.7	86.8-99.0	91.9	86.3-97.9
+37	75.6	70.8-80.8	73.6	67.4-80.5
+45	60.3	56.4-64.5	61.6	57.4-66.1
Predicted loss per year (%)	**0.010**		**0.055**	
Upper 95% CI of potency loss (%)	**0.018**		**0.098**	

Table 7. Bench stability following reconstitution.

Time following reconstitution at 4°C	% activity remaining (relative to freshly-opened ampoule)			
	Sample B (16/356)	95% CI	Sample C (16/358)	95% CI
4 h	101.7	95.9-107.8	97.8	89.0-107.6
8 h	96.9	91.4-102.7	101.4	92.2-111.5
24 h	99.3	93.7-105.3	101.7	92.5-111.8

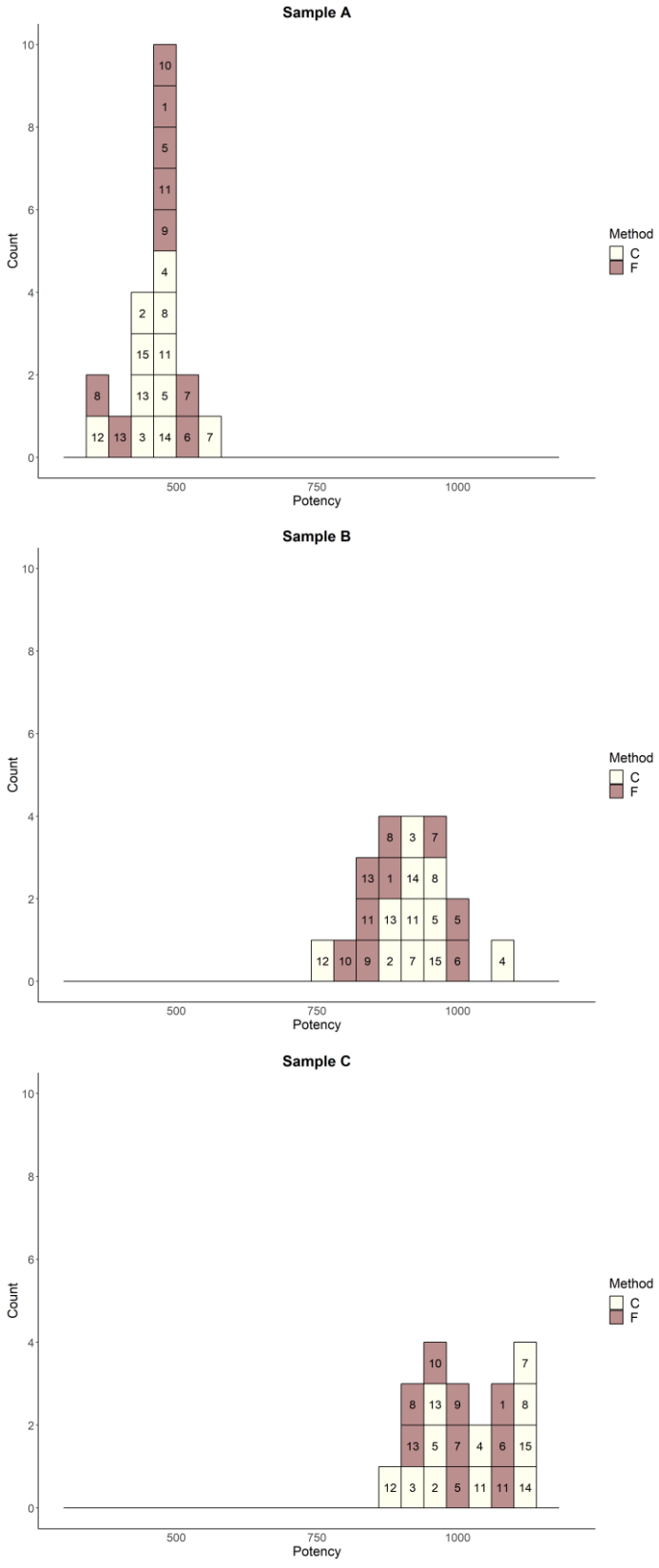
Table 8. Potency of sample A (88/824) against the 1st (62/7), 2nd (88/826), 3rd (00/464), and the candidate 4th IS for SK (16/358).

Study	Standard	Potency 88/824 (IU/ampoule)
1990	62/007	461
2000	88/826	469
2000	00/464	461
2018	00/464	461
2018	16/358 (1013 IU/ampoule)	460

Table 9. Potency estimates of sample A (88/824) relative to the candidate 4th IS for SK (sample C, 16/358, 1013 IU/ampoule).

Assay method	Lab	No. of assays	Potency (IU/ampoule)	Geometric Mean	Inter-lab GCV %
Clot lysis	1	4	451.12	**468.37**	**9.2**
	5	4	494.81		
	6	1	487.28		
	7	4	530.78		
	8	4	398.13		
	9	4	471.83		
	10	4	510.56		
	11	3	437.79		
	13	3	447.46		
Chromogenic	2	4	475.25	**453.10**	**7.8**
	3	3	483.99		
	4	4	455.49		
	5	4	478.51		
	7	4	511.95		
	8	4	434.82		
	11	3	464.76		
	12	4	398.72		
	13	3	457.17		
	14	1	422.49		
	15	4	413.71		
Overall:				**459.91**	**8.4**

Figure 1. Histograms summarising potency estimates of samples A, B, and C relative to sample S, the 3rd IS for SK (00/464). 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 shading represents the different assay methods used (C = chromogenic, F = fibrinolytic).



Appendix 1. Study protocol

International Collaborative Study to Establish the 4th IS for Streptokinase

Study protocol CS602

1. SAMPLES PROVIDED FOR ASSAYS

4 ampoules of each of the following samples are provided:

S 3rd International Standard (00/464), 1030 IU/ml

A Candidate test material, ~ 470 IU/ml

B Candidate test material, ~1000 IU/ml

C Candidate test material, ~1000 IU/ml

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.

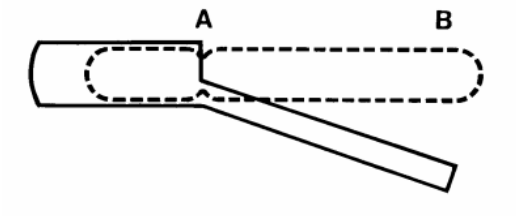
2. STORAGE AND RECONSTITUTION OF SAMPLES S, A, B AND C

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. 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 DIN ampoules (Samples S, B and C)
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

Directions for opening Sample B
Tap the ampoule gently to collect the material at the bottom (labelled) end. Ensure ampoule is scored all round at the narrow part of the neck, with a diamond or tungsten carbide tipped glass knife file or other suitable implement before attempting to open. Place the ampoule in the ampoule opener, positioning the score at position 'A'; shown in the diagram below. Surround the ampoule with cloth or layers of tissue paper. Grip the ampoule and holder in the hand and
squeeze at point 'B'. The ampoule will snap open. Take care to avoid cuts and projectile glass fragments that enter eyes. Take care that no material is lost from the ampoule and that no glass falls into the ampoule.



Side view of ampoule opening device containing an ampoule positioned ready to open. 'A' is the score mark and 'B' the point of applied pressure.

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 documents “Example of fibrin clot lysis assay used at NIBSC” and “Example of chromogenic assay used at NIBSC” provides example methods. If you want to repeat the study using more than one method we also encourage you to do so.

You are requested to carry out 4 independent assays (Assay 1-4), each using fresh ampoules of S, A, B, and C, over at least 2 days. For each assay, two independent dilution series from each ampoule should be prepared.

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. 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 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 no later than **27th October 2017**.

To calculate the potency of the test samples relative to the IS 00/464 raw data are required. Ideally we would like to receive time courses of absorbance changes if available.

Acceptable data formats for clot lysis assays are:

I. Time to 50% lysis of clots in all wells or tubes (including all replicates, and not just the means).

II. Raw data in the form of clot lysis profiles (a column of time versus columns of absorbance data) in txt or csv format, for example.

III. A Softmax Data file (.pda or .sda).

Acceptable data formats for chromogenic assays are:

I. Rates of plasminogen activation for initial absorbance changes (O.D. <0.2). These should be expressed as absorbance change per time squared (e.g. Abs/sec^2), or endpoint data if that is available.

II. Raw data in the form of plasminogen activation profiles (a column of time versus columns of absorbance data) in txt or csv 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 Streptokinase concentration used in nominal IU/ml and responses. Calculation of potency is optional, as this will be carried out at NIBSC.**

Data Analysis using Apps

Calculation of 50% lysis times and plasminogen activation rates can be facilitated using recently developed Apps, written in the open source language R and the Shiny Package, and may be used to analyse time course data (Longstaff, J Thromb Haemost 15, 1044-46, 2017).

The following links can be used to analyse data, with instructions provided in the "Help" tabs.
For clot lysis assay data: https://drclongstaff.shinyapps.io/clotlysisCL/
For chromogenic assay data: https://drclongstaff.shinyapps.io/zymogenactnCL/

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

Example of chromogenic assay used at NIBSC

ASSAY PRINCIPLE

tPA activates plasminogen to form the activate serine protease, plasmin. Fibrin is the usual substrate for plasmin, but in this assay plasmin activity is determined using the chromogenic substrate S-2251 The reaction mixture contains plasminogen, SK and S-2251, with the plasminogen activation step taking place in solution. Plasmin generation at a given streptokinase concentration is linear, with a resulting acceleration of S-2251 hydrolysis. The amount of p-nitroaniline formed, and the absorbance of the solution at 405 nm, increases exponentially, and is proportional to the SK concentration. The aim of the study is to assign potencies to three candidate samples (labelled A, B, and C). The potency of the test samples is determined by comparison with a reference preparation, in this case the 3rd International Standard for Streptokinase (code 00/464, Sample S), with an assigned potency of 1030 IU per ampoule.

MATERIALS

Buffers

A 0.5 M Tris from tables. 0.5 M Tris (30.29 g, 121.14 g/mol) + 183 ml of 1 M HCl made up to 500 ml (pH is 7.7 at RT). This solution is stable for several months at 4°C.

B Made from buffer A. Is 10 mM Tris HCl with 100 mM NaCl and 0.01% Tween 20 (5 ml of buffer A + 1.46 g NaCl and 250 µl of 10% Tween 20 up to 250 ml). Can be stored at 4°C.

C Buffer B + 1 mg/ml human (or bovine) albumin (500 µl of 20% albumin in 100 ml). Make fresh each day.

Human Plasminogen

A stock solution of glu-plasminogen, made up to 1 mg/ml in water. Aliquots stored at -40°C.

Plasmin chromogenic substrate

For example, S-2251 dissolved in distilled water to make a 3mM stock solution. Store at 4°C.

Streptokinase

Ampoules S, A, B, and C. Reconstituted in 1 ml distilled water.

S = 3rd International Standard (00/464), 1030 IU/ml

A = Candidate test material, ~470 IU/ml

B = Candidate test material, ~1000 IU/ml

C = Candidate test material, ~1000 IU/ml

Substrate Solution

Substrate solution is prepared by mixing 1.0 ml of Buffer A with 1.0 ml of 3 mM S-2251 and adding 5 µl 10% Tween 20. This solution is kept at 37°C in a water bath. Immediately before commencing the activation assay, 45 µl of 1 mg/ml plasminogen solution is added.

METHOD

Each assay requires at least two replicate dilution ranges of 4 doses of SK from each ampoule S, A, B, and C. All dilutions are carried out in **Buffer C.** The most concentrated solution is 4.0 IU/ml and a range of doubling dilutions is prepared from this solution. Two pre-dilutions may be performed to make the 4 IU/ml solution as follows:

Sample	1st pre-dilution	2nd pre-dilution	Nominal concentration (IU/ml)
S	50 µl -> 0.5 ml	39 µl -> 1 ml	4 IU/ml
A	110 µl -> 0.5 ml	39 µl -> 1 ml	4 IU/ml
B	50 µl -> 0.5 ml	39 µl -> 1 ml	4 IU/ml
C	50 µl -> 0.5 ml	39 µl -> 1 ml	4 IU/ml

The 4 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 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 SK pre-dilution (µl)	Buffer C (µl)	Nominal concentration (IU/ml)
D1	200	0	4
D2	100 (D1)	100	2
D3	100 (D2)	100	1
D4	100 (D3)	100	0.5

Using a multi-channel pipette, 60 µl of the SK dilution range (blue wells) is transferred to the corresponding wells in the lower half of the plate (as 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 40 µl the **substrate solution** to the wells containing the SK solutions using a multi-channel pipette (the final concentrations in the reaction mixtures are 2.4, 1.2, 0.6, and 0.3 IU/ml).

The plate is read kinetically at 37 °C for up to 1.5 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 O.D. of 0.1 (having adjusted the readings for the O.D. 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). Select for time squared for O.D. changes up to 0.1 arbitrary units to display rates in Abs/sec^2 (in the example below, ignore the settings below "select for time squared" and leave the "calculate rates in pM/s" box unchecked). However, if you choose to use this method, we would still like 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 here:

Analysis of zymogen activation

Select dataset:

Browse... 260717 SK 4th IS fills chromogenic2.csv

Upload complete

File type

- (•) csv
- ( ) txt

[x] Columns have header text

How many rows in your table?

8

Select data for second tab

A1

Select maximum number of points

75

Select maximum in absorbance

0.1

[x] Select to zero at initial absorbance

[x] Select for time squared

[ ] Calculate rates in pM/s using constants below

kcat (s-1) for chromogenic substrate

60

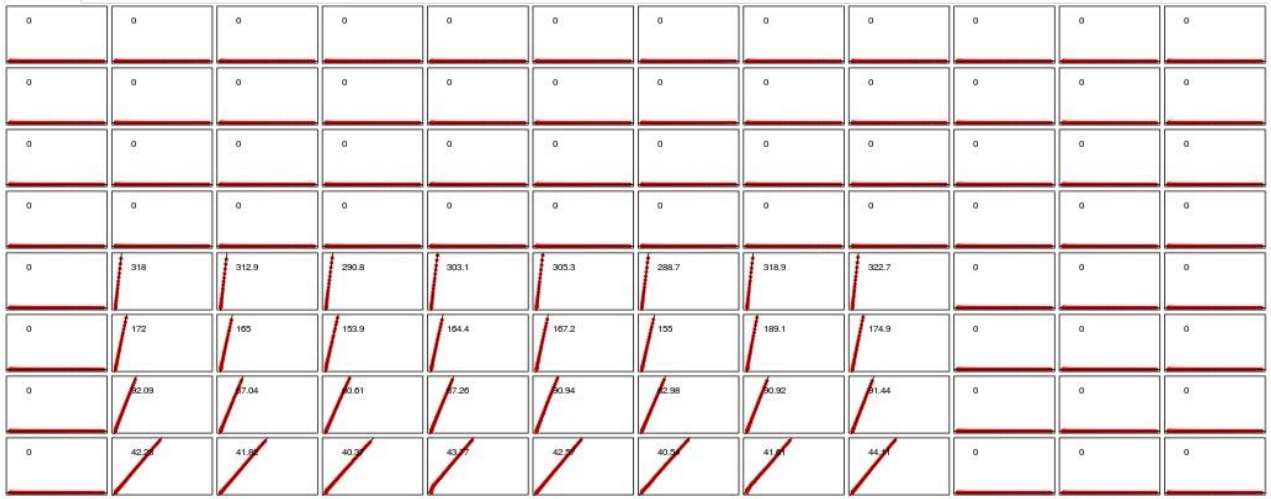
Km (mM) for chromogenic substrate

0.26

[S] (mM) for chromogenic substrate

0.24

Plots | Curve | Raw data | Help



Rates Abs/s² x 1e9 for maximum absorbance 0.1 and 75 data points

V1	V2	V3	V4	V5	V6	V7	V8	V9	V10	V11	V12
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.00	318.01	312.86	290.78	303.07	305.33	288.72	318.95	322.73	0.00	0.00	0.00
0.00	172.05	164.99	153.86	164.40	167.21	155.02	189.11	174.90	0.00	0.00	0.00
0.00	92.09	87.04	80.61	87.26	90.94	82.98	90.92	91.44	0.00	0.00	0.00
0.00	42.23	41.82	40.37	43.77	42.57	40.54	41.62	44.11	0.00	0.00	0.00

Ignore these settings

Example of fibrin clot lysis assay used at NIBSC

ASSAY PRINCIPLE

Fibrinogen is clotted with thrombin in a 96-well plate, in a reaction mixture also containing streptokinase and plasminogen, so that clotting is immediately followed by lysis. Both phases can be monitored by changes in turbidity using a plate reader. The time to 50% lysis of the clot is used as the endpoint, which corresponds to the time taken for the maximum absorbance to decrease by half. Lysis is proportional to the rate of plasmin generation, and therefore the amount of streptokinase in the starting mixture. The aim of the study is to assign potencies to three candidate samples (labelled A, B, and C). The potency of the test samples is determined by comparison with a reference preparation, in this case the 3rd International Standard for Streptokinase (code 00/464, Sample S), with an assigned potency of 1030 IU per ampoule.

MATERIALS

Buffers

A 0.5 M Tris from tables. 0.5 M Tris (30.29 g, 121.14 g/mol) + 183 ml of 1 M HCl made up to 500 ml (pH is 7.7 at RT). This solution is stable for several months at 4°C.

B Made from buffer A. Is 10 mM Tris HCl with 100 mM NaCl and 0.01% Tween 20 (5 ml of buffer A + 1.46 g NaCl and 250 µl of 10% Tween 20 up to 250 ml). Can be stored at 4°C.

C Buffer B + 1 mg/ml human (or bovine) albumin (500 µl of 20% albumin in 100 ml). Make fresh each day.

D Buffer C + thrombin (5 µl of 100 IU/ml (or 1 µM) thrombin per ml of Buffer C). Make fresh each day.

Human Plasminogen

A stock solution of glu-plasminogen, made up to 1 mg/ml in water. Aliquots stored at -40°C.

Fibrinogen

One bottle of 0.5 g fibrinogen (from Calbiochem, for example) is dissolved in 10 ml of buffer B at RT. 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.

Fibrinogen + Plasminogen Solution

A 0.5 ml aliquot of fibrinogen solution (50 mg/ml) is thawed and made to 3.6 mls with Buffer B, and kept at 37 °C. Immediately before clotting is to begin, 75 µl of glu-plasminogen, 1 mg/ml, is added.

Thrombin

Use a working stock solution of 100 IU/ml (around 1 µM). Can be human or bovine. Assume IU are approximately equivalent to N.I.H. units if they are used.

Streptokinase

Ampoules S, A, B, and C. Reconstituted in 1 ml distilled water.

S = 3rd International Standard (00/464), 1030 IU/ml

A = Candidate test material, ~470 IU/ml

B = Candidate test material, ~1000 IU/ml

C = Candidate test material, ~1000 IU/ml

METHOD

Streptokinase + Thrombin solution

Each assay requires at least two replicate dilution ranges of 4 doses of streptokinase (SK) from each ampoule S, A, B, and C. All dilutions are carried out in **Buffer D**, which contains thrombin**.** The most concentrated solution is 1 IU/ml and a range of doubling dilutions is prepared from this solution. Two pre-dilutions may be performed to make the 1 IU/ml solution as follows:

Sample	1st pre-dilution	2nd pre-dilution	Nominal concentration (IU/ml)
S	25 µl -> 0.5 ml	20 µl -> 1 ml	1 IU/ml
A	55 µl -> 0.5 ml	20 µl -> 1 ml	1 IU/ml
B	25 µl -> 0.5 ml	20 µl -> 1 ml	1 IU/ml
C	25 µl -> 0.5 ml	20 µl -> 1 ml	1 IU/ml

The 1 IU/ml dilution (designated D1) is then used to make three additional doubling dilutions (D2-D4) in **Buffer D**, 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 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 SK pre-dilution (µl)	Buffer D (µl)	Nominal concentration (IU/ml)
D1	200	0	1
D2	100 (D1)	100	0.5
D3	100 (D2)	100	0.25
D4	100 (D3)	100	0.125

Using a multi-channel pipette, 60 µl of the SK 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			

Fibrinolysis

The reaction is initiated by adding 40 µl of fibrinogen/plasminogen solution to the wells containing the 60 µl SK solutions as quickly as possible (the final concentrations of SK in the reaction mixtures are 0.6, 0.3, 0.15, and 0.075 IU/ml). The solutions and the plate should be pre-warmed to 37°C. The plate is read kinetically at 405 nm at 37 °C for up to 2 hours or until the clot is completely lysed. Reactions should not need to be monitored for > 5 hours to avoid drying out.

Data reporting

Clot lysis time is recorded as the time taken for 50% lysis 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/clotlysisCL/). However, if you choose to use this method, we would still like 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:

Analysis of clotting and lysis data

Select dataset:

Browse... 260717 SK 4th IS... c

Upload complete

File type

- csv
- txt

Columns have header text

How many rows? 8

% Clot formed or remaining (0.5 is 50%) 0.5

x axis range

0 1,984 25,000

0 2,500 5,000 7,500 10,000 12,500 15,000 17,500 20,000 22,500 25,000

y axis range

-1.5 0 0.45 2.5

-1.5 -1.1 -0.7 -0.3 0.1 0.5 0.9 1.3 1.7 2.1 2.5

Baseline Options

- global zero
- nth absorbance
- min abs + offset below

global zero 0.048

nth point 1

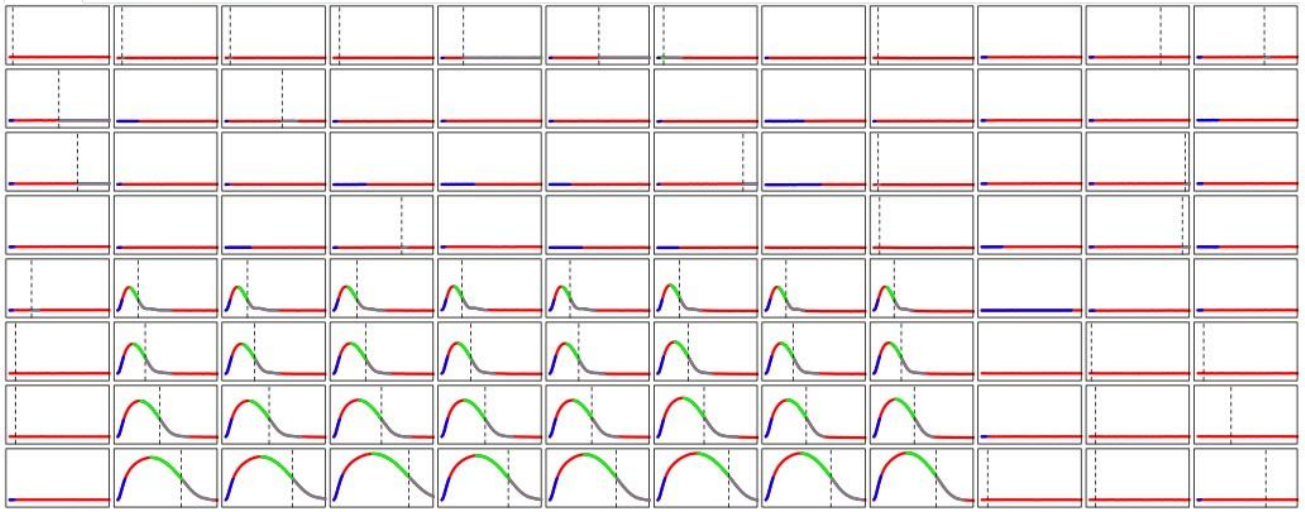
-0.15 0 0.15

-0.15 -0.12 -0.09 -0.06 -0.03 0 0.03 0.06 0.09 0.12 0.15

Results Table

- Column names
- Max abs
- Max abs - zero
- Time to max
- Time to clotting
- Time to chosen lysis
- Time between clotting and lysis
- Time to lysis from peak

Plots | Curve | Raw data | Help



Time to chosen lysis for 50 % clot formation or clot remaining

Results Table

	1	2	3	4	5	6	7	8	9	10	11	12
1	60	90	90	120	450	1020	120	4320	90	3210	1470	1380
2	1020	2220	1170	4320	6030	3750	4710	3240	3540	5220	5220	6540
3	1410	4800	3240	3540	2370	3480	1770	6690	90	2370	1980	4260
4	6540	4260	2310	1410	2700	5370	6540	6540	120	5220	1920	6450
5	450	420	450	480	420	420	450	420	420	6030	4260	2700
6	120	570	600	660	600	600	630	570	570	4260	30	120
7	120	870	900	990	900	870	960	840	840	6450	120	690
8	3540	1320	1380	1560	1380	1380	1470	1350	1290	120	120	1410

Calibration of the proposed WHO 4th International Standard for Streptokinase

CS602: Results sheet

Laboratory:

Name:

Method: *Please provide brief details e.g. equipment used, chromogenic substrate, measurement parameters. If lysis times are recorded, please outline the method and endpoint used. 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 another 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. clot lysis time or rate) rather than as % or units relative to an in house standard.

EXAMPLE

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

		RESULT Time to 50% lysis in seconds							
	Nominal conc. (IU/ml)	**S**	**A**	**B**	**C**	**C'**	**B'**	**A'**	**S'**
D1	**1**	780	810	900	810	810	900	810	810
D2	**0.5**	1200	1200	1410	1230	1170	1380	1230	1230
D3	**0.25**	1890	1920	2220	1920	1890	2220	1950	1950
D4	**0.125**	2970	3060	3420	3060	2940	3450	3150	3030

Pre-dilution information:

To obtain the 1 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	25 µl -> 0.5 ml	20 µl -> 1 ml	1
A	55 µl -> 0.5 ml	20 µl -> 1 ml	1
B	25 µl -> 0.5 ml	20 µl -> 1 ml	1
C	25 µl -> 0.5 ml	20 µl -> 1 ml	1

Day 1

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			

Day 1

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			

Day 2

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			

Day 2

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.

Marco Müsken BSV Bioscience GmbH Max-Planck-Strasse 12 Baesweiler 52499 Germany	Nicola Mutch University of Aberdeen School of Medicine, Medical Sciences and Nutrition Institute of Medical Sciences Foresterhill, Aberdeen AB25 2ZD, UK	Vishwanath Malkar/Sandeep Gangurde Bharat Serums & Vaccines Limited Anand Nagar Ambernath (E) Maharashtra 421 501, India
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Appendix 3. Participants' Response Sheet

Medicines & Healthcare products Regulatory Agency

NIBSC
Confidence in Biological Medicines

An international collaborative study to establish the WHO 4th International Standard for Streptokinase

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 16/358 is proposed as the WHO 4th International Standard for Streptokinase with a potency of 1013 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 16/358. Please respond below if you would like to take up this offer.

☐ Yes, I would like 6 ampoules of 16/358

☐ No, I would not like 6 ampoules of 16/358

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

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World Health Organization