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An International Collaborative Study to Establish the 2nd International Standard for Activated Factor IX (FIXa)

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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 **18 September 2017** 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 C M Nübling** at email: **nueblingc@who.int**

Summary

Nineteen laboratories participated in the value assignment of the 2nd International Standard for FIXa (NIBSC code, 14/316) relative to the 1st International Standard for FIXa (NIBSC code, 97/526). Participants submitted 17 sets of data from purified reagent chromogenic/fluorogenic based assays, 9 sets from one-stage clotting assays based on APTT (8 different APTT reagents), 2 sets of one-stage clotting assays based on NAPTT (2 different phospholipid reagents) and 6 sets based on thrombin generation test (TGT). The candidate preparation was provided as coded duplicates (samples A and B) and with the exception of one laboratory, all participants obtained similar potencies for the coded duplicates. The overall geometric mean (GM) potency for the candidate preparation by purified reagent assays was 10.48 IU/ampoule (inter-laboratory GCV of 4.68%, majority of intra-laboratory GCVs <10%). The overall GM potencies by APTT and NAPTT were higher at respectively 11.67 and 12.10 IU/ampoule. TGT results from one laboratory gave similar potencies to GM obtained from the purified reagent assays. Sample C, a FIX concentrate with low level of FIXa was included in the study and results indicate the purified reagent assays were sufficiently sensitive to detect low level of FIXa (GM= 0.015 IU/ml) in large amount of zymogen FIX. However, not all the participants were able to obtain statistically valid assays suggesting a need for optimisation of these assays. In addition, the majority of the intra-laboratory GCVs were <10%, the inter-laboratory GCV of 35% was high indicating the need to improve inter-laboratory agreement for these purified reagent assays. The markedly higher GMs, 3.10 and 0.27 IU/mL obtained respectively by APTT and NAPTT for sample C demonstrate the unsuitability of these two clot-based assays for the measurement of FIXa in FIX concentrates. TGT using tissue factor only as trigger gave estimates for sample C similar to GM from purified reagent assays, but higher values were obtained when FXIa was used in combination with tissue factor as the activator. Because of the uncertainty of the influence of other components involved in clot and plasma based assays on the measurement of FIXa and that the 1st International Standard for FIXa was labelled based on consensus mean from purified reagent assays only, it is proposed to value assign the replacement standard with GM obtained by purified reagent assays only. This proposal was agreed by the participants of the study and it is therefore recommended that 14/316 be established as the 2nd International Standard for FIXa with a labelled potency of 10.5 IU/ampoule.

Introduction

The 1st International Standard for Factor IXa, 97/526 was established in 1998 and its main use is for measurement of activated factor IX (FIXa), a highly thrombogenic process related impurity found in therapeutic prothrombin complex concentrates (PCCs) and monocomponent plasma derived and recombinant FIX concentrates. Recently, there has been an increase in the demand for this International Standard as new generation modified FIX products are now licensed for replacement therapy. As the stock of this International Standard is now close to exhaustion and there is no secondary standard available, a replacement standard is urgently required.

In the present study, a candidate preparation, prepared by the same process as for the bulk starting material of the 1st International Standard (IS) has been value assigned relative to the 1st IS, with a view to establish this material as the 2nd IS for FIXa.

Candidate WHO 2nd International Standard for Activated FIX (FIXa) (14/316)

The bulk starting material for the candidate preparation, 14/316 was kindly provided by Pfizer, Andover, Massachusetts, US and was prepared by FXIa activation of human recombinant FIX. The purity of the FIXa was assessed and confirmed by PAGE with silver staining. The estimated specific activity of the bulk was 612 IU/mg. Sixy-two ml of the frozen bulk was thawed at 37°C diluted in buffer (0.05MTris, 0.15M NaCl, 5 mg/ml trehalose, 1.25% human albumin, pH 7.4) to approximately 10 IU/ml. The solution was distributed into approximately 18,000 ampoules at 4°C and freeze-dried. The finished product characteristics are as follows:

Code number	14/316
Presentation	Sealed, 3 ml glass ampoules
Number of ampoules available	18000
Date filled	23 April 2015
Mean fill mass (n=410)	1.0083 g
Precision of fill (CV of fill mass) (n=675)	0.169%
Residual moisture (n=12)	0.152%
Mean dry weight (n=6)	0.0303g
Mean oxygen head space (n=12)	0.34 %
Storage conditions	-20°C
Address of processing facility	NIBSC, Potters Bar, EN6 3QG, UK
Address of custodian	NIBSC, Potters Bar, EN6 3QG, UK

Participants

Twenty laboratories agreed to take part in the study and 19 participants (5 Austria, 1 Canada, 1 China, 3 France, 4 Germany, 1 The Netherlands, 1 Sweden, 1 UK, 2 USA) returned data for analysis. Each participant is referred to in this report by an arbitrarily assigned number, not representing the order of listing in Appendix I.

Samples

Four coded samples were included in the study. The 1st International Standard for Activated Factor IX (FIXa) (97/562), with a labelled potency of 11 IU/ampoule was coded S. The candidate preparation (NIBSC 14/316) was provided as coded duplicates, samples A and B and each ampoule contains approximately 10 IU of FIXa. Sample C was a FIX concentrate containing approximately 10 IU/ml of FIX and approximately 0.01 IU/ml of FIXa.

Assay methods

Each laboratory was asked to perform their in-house method(s) for FIXa. Multiple result sets returned by a participating laboratory were treated as results from an independent laboratory and were given a separate lab code, e.g. Lab 2a, Lab 2b. Table 1 shows the assay methods used by the participants. Participants submitted 17 sets of data from assays using purified reagents based on VIIIa activation and Xa generation as end point (16 sets using chromogenic substrates, 1 set using fluorogenic substrate), 9 sets from one-stage clotting assays based on APTT (8 different APTT reagents), 2 sets of one-stage clotting assays based on NAPTT (2 different phospholipid reagents) and 6 sets based on thrombin generation test (TGT).

Study design

Details of the assay design were as stated in the protocol which is attached as Appendix II. Briefly, each participant was requested to carry out 4 independent assays on 4 sets of samples and to follow one of the suggested balanced assay designs as described in the study protocol.

Statistical analysis

The potencies of all samples were calculated relative to the 1st International Standard for Factor IXa 97/562 (coded S) by parallel line analysis of the raw assay data (Finney 1978). All chromogenic assays were analysed with a log transformation of the assay response and the majority of clot-based assays without any transformation (except labs 10, 12a, 18 and 19 which were log transformed). The fluorogenic assays completed by lab 03 were log transformed. Lab 08 returned TGT assay results, using tissue factor with and without FXIa; measuring clot time, Thrombin peak height (TPH), and Time to Peak (TTP). The TTP without FXIa and TPH with FIXa were log transformed.

For all assays linear parallel line analysis was performed, using a linear section of the response range with a minimum of three dilutions for all samples. Calculations were performed using the EDQM software CombiStats Version 5.0.

Non-linearity and non-parallelism were considered in the assessment of assay validity. Samples were visually inspected for non-linearity in all cases. Non-parallelism was assessed by calculation of the ratio of fitted slopes for the test and reference samples under consideration. The samples were concluded to be non-parallel when the slope ratio was outside of the range 0.80 – 1.25 and no estimates are reported.

Relative potency estimates from all valid assays were combined to generate an unweighted geometric mean (GM) for each laboratory and these laboratory means were used to calculate an overall unweighted geometric mean for each sample and reagent. Variability between assays within laboratories and between laboratories has been expressed using geometric coefficients of variation (GCV = $\{10^{s}-1\}\times100\%$ where s is the standard deviation of the log10 transformed

estimates) (Kirkwood 1979). Comparisons between methods were carried out by the relevant t-test of log potencies.

Results

Assay Data
The 19 participants contributed data from a total of 141 assays: 64 chromogenic, 4 fluorogenic, 35 APTT based one-stage clotting, 8 NAPTT based one-stage clotting, 30 sets thrombin generation type assays.

Assay Validity
Details on exclusion of assays based on assay validity criteria as described in the Statistical analysis section are shown in Appendix III. Appendix III A also shows a comparison of laboratory's own and NIBSC's estimated potencies for each sample by chromogenic, fluorogenic, APTT and NAPTT assays, while Appendix III B provides brief description of reagents used and detailed potencies and obtained by thrombin generation assay as performed by Lab 8.

Potency estimates, intra- and inter-laboratory variability
Samples A and B
The potency estimates for samples A and B relative to the 1st IS for FIXa, the variability within each laboratory and between laboratories for purified reagent assays (chromogenic and fluorogenic based methods) expressed as geometric coefficients of variation (% GCVs) are given in Table 2. The laboratories' overall potency estimates are also illustrated as histograms (Figure 1). Since samples A and B were coded duplicates, the potencies for these 2 samples should be similar within an assay, yielding a potency ratio of A/B close to 1. Based on results from this study where the majority of the A/B potency ratios were found to be within the range of 0.9 -1.1, laboratory results, where the potency ratios were outside this range were excluded from the calculation of the overall potency estimates. Based on this criterion, results from Lab 8a and Lab 8b were excluded. The overall potencies, excluding Lab 8a and Lab 8b were 10.60 IU/mL and 10.48 IU/mL for samples A and B respectively. With the exception of Lab 4, Lab 8a, Lab 8b and Lab 17a for sample A, and Lab 7, Lab 17b and Lab 18a for sample B, the majority of intra-laboratory GCV were <10%. The ranges were 1.19 – 17.50% for sample A and 2.55 – 12.40% for sample B. Excluding results from Lab 8a and 8b, the inter-laboratory variability expressed as GCVs for samples A (4.13%) and B (4.84%) were similar. Paired t-test of log geometric mean potencies indicated that there was no significant differences between the potency estimates for samples A and B ($p = 0.822$). The estimates for samples A and B were therefore combined to yield an overall potency of 10.48 IU/mL for the candidate preparation, 14/316 (Table 3). Table 3 also shows the intra-laboratory variability for combined samples A and B and the GCVs ranged from 1.19 to 11.16%. The inter-laboratory GCV, excluding results from Lab 8a and Lab 8b was 4.68%.

Table 4 shows intra-laboratory %GCVs for APTT based assays ranged from 2.11 to 7.90 and 1.37 to 8.88 for samples A and B respectively, with inter-laboratory GCVs at 15.76% for sample A and 15.19% for sample B. The ratios of A/B were all within 0.9 – 1.1. The overall potency

estimates were 11.65 IU/mL and 11.80 IU/mL for samples A and B respectively. The results are also shown in Figure 2. Paired t-test of the log geometric mean potencies (Lab 12a results excluded from analysis as no valid estimates was obtained for sample A) showed that there was no significant difference between estimates for samples A and B ($p = 0.626$). The potencies for samples A and B were therefore combined. As shown in Table 5, the intra-laboratory GCVs for combined samples A and B ranged from 0.89% to 8.25% for APTT, with corresponding inter-laboratory GCV of 16.20%. The overall potency was 11.67 IU/mL. For NAPTT clot based method, the intra-laboratory GCVs were <8% for both samples (Table 4) and GCVs for the 2 laboratories were 6.52% and 2.79% (Table 5) when the results for samples A and B were combined. Inter-laboratory GCVs were not calculated as there were only results from 2 laboratories. The overall potency by NAPTT was 12.10 IU/mL which was similar to the individual estimates of 12.20 IU/mL for sample A and 11.98 IU/mL for sample B.

Only one participant used thrombin generation test (TGT) to estimate potency of the candidates and results obtained using different triggers (tissue factor only and tissue factor and FXIa) are listed as for Lab 8a and Lab 8b in Tables 8a and 8b. Similar potency estimates using different readouts from the same assay were observed and also similar ranges to those obtained with the purified reagent assays (Tables 2 and 8a/8b). Overall higher intra-laboratory GCVs were obtained for TGT assays using tissue factor only trigger by comparison with GCVs from tissue factor/ FXIa assays.

Sample C

Tables 6 and 7 show the intra-laboratory variability for sample C, a FIX concentrate with low level of FIXa, by purified reagent and clot-based methods respectively. Figure 3 also presents potency estimates for sample C by these methods. For the purified reagent assays, only 11 out of the 17 participants were able to obtain statistically valid estimates of FIXa. With the exception of Lab 8, which obtained GCV >17%, the intra-laboratory GCVs were reasonably low, ranging from 1.16% to 9.45%. However, an inter-laboratory GCV of 35.17% indicates poor between laboratory agreement of potencies. For clot-based assays, the range of intra-laboratory GCV was 3.44 % - 9.17% and inter-laboratory GCV at 33.41% was similar to that observed for the purified reagent assays. In terms of potencies, the purified reagent assay gave estimates ranging from 0.010 – 0.029 IU/mL, with an overall potency of 0.015 IU/mL. However, values obtained by the clot based APTT assays were markedly higher, ranging from 2.44 IU/mL to 5.29 IU/mL (overall potency of 3.10 IU/mL). Although the estimate by NAPTT was lower at 0.27 IU/mL (results only from one laboratory), this is still much higher than that obtained by the purified reagent assays.

Similar results to the purified reagent assays by TGT were obtained for sample C (Tables 8a and 8b). However, the majority of the assays were invalid, suggesting that the TGT in the formats carried out by Lab 8a and 8b were not sufficiently robust to measure low levels of FIXa in FIX concentrates.

Stability Studies

Accelerated degradation study

Table 10a presents results from the accelerated degradation study carried out at 4 time points. The activity of the samples stored at elevated temperatures were compared to activity of the preparation stored at -150◦C. This candidate preparation, 14/316 showed high degree of stability and has no predicted loss of activity at the storage temperature of -20°C.

On-bench stability

On-bench stability of candidate, 14/316 was assessed using a FIXa functional chromogenic assay. An ampoule of the candidate preparation was reconstituted and stored on melting ice. Two independent assays were carried out on an aliquot at hourly intervals against a freshly reconstituted ampoule of 14/316. The results in table 10b showed that the activity did not change over the 4 hour period as supported by the overlapping 95% confident limits of the assays. This indicates the reconstituted ampoules would be stable for up to 4 hours when stored on melting ice.

Discussion

The main aim of this study was to value assign a replacement International Standard for FIXa. In addition, a FIX concentrate with low level of FIXa was included in the study to evaluate the validity of using a purified preparation of FIXa as a reference standard to measure FIXa, a process related impurity in FIX therapeutic concentrates. The inclusion of the coded duplicates (samples A and B) of the candidate preparation, 14/316 allowed the assessment of both laboratory and assay method performance. The 19 participants returned data from purified reagent methods which included a selection of commercial chromogenic assay kits, in-house chromogenic and fluorogenic assays and clot based methods which included APTT assays involving a variety of APTT reagents and NAPTT assays. One laboratory returned data for TGT with multiple readouts. The TGT is a plasma based assay and this participant employed tissue factor only and tissue factor with FXIa as triggers.

Of the 17 sets of data from the purified reagent assays, only one laboratory (Lab 13a) obtained statistically invalid assays, due mostly to non-linearity of standard curves. With the exception of 2 labs (Lab 8a and 8b), the close to 1 potency ratios of A/B indicated the laboratories were able to perform the assays with little bias. In addition, the low intra-laboratory %GCVs indicate the laboratories were able to perform these chromogenic and fluorogenic assays with good precision. The inter-laboratory GCV of 4.68% was also low, showing that there was good agreement of potencies between laboratories. Two chromogenic commercial kits (Rossix and Hyphen Biomed) were employed by the participants and a 2-sample t test of the log potencies of samples A and B combined by these 2 methods indicated that there was no significant differences between the estimates ($p = 0.559$). The overall potency estimate for the candidate preparation (14/316), obtained relative to the 1st International Standard for FIXa by pooling results for samples A and B was 10.48 IU/mL.

As exemplified by the low intra-laboratory %GCVs, the participants were also able to perform the clot-based methods for samples A and B with good precision; however, the inter-laboratory variability was much higher at 16.20% by comparison with 4.68% calculated for the purified reagent assays. As shown in Table 9, the clot based methods gave ~11 and 15% higher potencies by APTT and NAPTT respectively compared with the value obtained by the purified reagent assays. However, the difference between the purified reagent assays and the APTT was not

statistically significant (2 sample t-test of log potencies, p = 0.153). For the TGT, the different readouts gave reasonably similar estimates which were also close to results for samples A and B from the purified reagent assays, suggesting that this plasma based assay is more specific than the clot-based APTT and NAPTT. However, in general, the intra-laboratory %GCVs, especially for assays triggered by tissue factor alone were higher than those obtained for the purified reagent assay.

The major intended use of the IS for FIXa is to harmonise the measurement of FIXa, a process related impurity in FIX concentrates. Sample C, a FIX concentrate with low level of FIXa was therefore included in the study to investigate the assay and reference standard performance. For the purified reagent assays, some laboratories were unable to obtain statistically valid assays for sample C and this was due mostly to dose response curves being out of range of the standard curves and/or lack of regression of the dose responses at low concentrations of FIXa. It may be possible for these laboratories to obtain valid assays if they adjust the doses used for the assay of sample C. The intra-laboratory %GCVs were in similar ranges to those obtained for samples A and B indicating the laboratories' capability of measuring low levels of FIXa in FIX concentrates with reasonable precision. The inter-laboratory variability of >30% was much higher than that obtained for samples A and B. The majority of the laboratories used commercial assay kits and there was no obvious assay discrepancy. It is possible that further refinement and harmonisation of variability such as pre- and post- analytical parameters may help to improve inter-laboratory agreement. The APTT gave up to 200 fold higher estimates (3 IU/ml by APTT, 0.015 IU/ml by purified reagent assays) than the purified reagent methods. One laboratory obtained 2 valid NAPTT assays for sample C and although the 2 estimates were in reasonably good agreement (appendix IIIA, Lab 18d), the overall potency was still 18 fold (0.27 IU/mL) higher. The higher potencies were probably due to the influence of unactivated FIX in these assays. This indicates these clot-based methods are not suitable for assessment of FIXa in FIX concentrates. Interestingly, the TGT using tissue factor alone as the activator did give similar estimates of sample C to the purified reagent assays, but the values obtained using tissue factor and FXIa as trigger was close to results from the NAPTT, but higher than the purified reagent assay estimate. This is possibly a reflection of the differential specificity and sensitivity of the two triggers used. It appears that the participant had adjusted the concentrations of the triggers so that the FXIa in the tissue factor and FXIa combination may have promoted the activation of zymogen FIX present in sample C. While all the assays (5) from the Time to Peak (tissue factor only) and Peak Thrombin (tissue factor and FXIa) were statistically valid, the majority of the other readouts for sample C were invalid. TGT using tissue factor alone could be a useful test for FIXa and further refinement and development of TGT may help to improve performance of this assay.

In the previous study for the value assignment of the 1st International Standard for FIXa, results from purified reagent chromogenic assays only were used for the consensus mean (Gray and Walker, 1998). The one laboratory that used APTT gave results substantially higher than the chromogenic assays and was excluded in the final calculation. The results from this study confirmed that clot-based assays can generate higher FIXa estimates than the purified reagent chromogenic and fluorogenic methods and this is markedly apparent in the results obtained for sample C, a FIX concentrate with low level of FIXa. It is therefore proposed to value assign the candidate preparation, 14/316 using results from purified reagent assays only.

Conclusion and Recommendation

Based on the consensus geometric mean from purified reagent assays relative to the 1st International Standard for FIXa, 97/562, it is recommended that the candidate preparation, 14/316, be established as the 2nd International standard for FIXa with an assigned potency of

10.5 IU/ampoule

Draft Instruction for Use

Appendix IV shows a draft of Instruction for Use for the 2nd International Standard for Activated FIX (FIXa).

Participants responses

All the participants who responded (17/19) agreed with the proposal and recommendation that 14/316 be established as the 2nd International Standard for FIXa with an assigned potency of 10.5 IU/ampoule.

Apart from minor typos which have been corrected, there were comments from 2 participants:

Participant 1

- Comment 1: We have noticed that the results calculated by NIBSC differ from our own calculations. In order to understand these differences, I would like to ask, if possible, to deliver us with your CombiStats analyses of our data.

o NIBSC response: Your returned results use a slope ratio model rather than a parallel line model used in our analysis which has caused the discrepancy in calculated potencies

- Comment 2: The statistical analysis section considers non-linearity and non-parallelism. Linearity is checked by visual inspection only. This might be a rather subjective measure for detecting non-linearity and should be avoided as sole measure. Additionally the test for non-linearity in the CombiStats ANOVA output should also be taken into account (p-value >=0.05 for no deviation from linearity; though it is acknowledged that this is not a formal proof for linearity).

Regarding non-parallelism the statistical analysis section states that the ratio of slopes must lie in the range of 0.80 and 1.25. This might also be too liberal and either the test for non-parallelism (p-value>=0.05) should be used or the 90% confidence interval for the ratio of slopes (also provided within the CombiStats output) should be in a reasonable (pre-specified) range."

o NIBSC response: We agree with the comments made regarding the statistical analysis, in particular regarding the subjective nature of the non-linearity assessment and the possibility that

the non-parallelism approach may be too liberal. Ideally we would apply an ANOVA approach or a non-parallelism assessment that uses the 90% confidence intervals on the slope ratios, but this raises difficulties in the context of a collaborative study where the quality of data and assay variability is very different in different laboratories. We therefore felt that the approach taken was appropriate to apply consistently to all laboratories and would not impact the outcomes and conclusions from the study. With regard to non-parallelism, we investigated two ranges which have been used in other clotting factor studies, 0.80-1.25 and 0.90-1.11. If we were to use the second range then 50% of samples in clotting assays and 20% of samples in chromogenic assays would have been excluded in addition to all non-linear samples and those out of range of the standard.

Participant 2

The description of Sample C assessment by the TGT method (last paragraph of section Sample C on page 5 and last three lines on page 6) should be revised to clarify the following:

1. The statement "Similar results to the purified reagent assays by TGT were obtained for sample C" should be corrected to clarify the differences between the tissue factor (TF) and TF and FXIa activated TGT methods.

Specifically, sample C potency by the TF-activated TGT (~ 0.013 IU/mL according to Appendix III B, Lab 8 Tissue factor initiated, time to peak) was in a good agreement with purified reagent assays. In contrast, sample C potency by the TF and FXIa-activated TGT (~ 0.2 IU/mL according to Appendix III B, Lab 8 Tissue factor-XIa initiated, thrombin peak height) was in a good agreement with the NAPPT assay.

The observed difference is likely related to the different sensitivities of the TF and TF/FXIa-activated TGT assay variants to the two proteins, FIX and FIXa, in the sample C. Under the dilutions used, the TF-activated TGT is almost exclusively sensitive to the FIXa activity. In contrast, FXIa-activated TGT assay is also sensitive to FIX, which is not surprising because FXIa promotes activation of FIX into FIXa under the assay conditions tested.

2. The statement ".. the majority of the assays were invalid, suggesting that the TGT … was not sufficiently robust to measure low levels of FIXa in FIX concentrates…" should be revised to highlight the observation that the TGT's performance was not much worse, and probably even better, than that of the classical NAPTT assay.

Indeed, all five assay runs were valid for the two TGT conditions: Appendix III B, Lab 8 Tissue factor initiated, time to peak; and Appendix III B, Lab 8 Tissue factor-XIa initiated, thrombin peak height. In contrast, only two out four assay runs were valid for the NAPPT assay, see Appendix III A: Lab 18d / Clot / NAPTT.

Off note, the thrombin peak height parameter was the most robust for the FXIa-activated TGT because the concentrations of the TF and FXIa triggers in this assay were specifically optimized to extend the linear range of the thrombin peak height (rather than, e.g., time to peak) responses to FIX/FIXa.

o NIBSC response: The text in the discussion has been revised to reflect this participant's comments. It should be noted that only 2 labs returned data for NAPTT and 1 lab provided results for TGT, there is insufficient data to fully appreciate the performance of these 2 assays in the measurement of FIXa, especially FIXa in FIX concentrate.

Scientific and Standardization Committee (SSC) Expert responses

All SSC nominated experts agreed with the proposal and recommendation. The SSC endorsed the establishment of this standard at the 63rd SSC meeting (Berlin, Germany) in July 2017.

References

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Acknowledgments

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Table 1: Methods used by the participants

Lab	Chromogenic Assays	Fluorogenic Assays	Clot-based Methods (method, reagent)	Thrombin Generation Test
1	Rossix			
2	Rossix			
3		In house (Xa generation)		
4a	Rossix			
4b			APTT, Actin-FS	
5			APTT, CK-Prest 2	
6	Biophen			
7	Biophen			
8a	Biophen			
8b	Rossix			
8c			APTT, SynthAFax	
8d				TF + XIa, IX def, Peak Height
8e				TF , IX def, Peak Height
8f				TF + XIa, IX def, tt Peak
8g				TF , IX def, tt Peak
8h				TF + XIa, IX def, clot-time
8i				TF , IX def, clot time
9			NAPTT, CK Prest2	
10			APTT, ApTT-SP	
11	In-house, pefachrome IXa			
12a			APTT, Dapttin	
12b			APTT, Siron LS	
13a	Biophen			
13b			APTT, APTT-SP	
14	Rossix			
15	Rossix			
16			APTT, Triniclot Automated APTT	
17a	Biophen			
17b	Rossix			
18a	In-house, Xa generation			
18b	Rossix			
18c	Hyphen			
18d			NAPTT, Rossix Phospholipids	
19			APTT, PTTA	

Table 2: Purified reagent assays - Laboratory's geometric mean (GM) and Intra-laboratory variation expressed as % geometric coefficient of variation (%GCV) and overall GM and inter-laboratory GCV for samples A and B, the coded duplicates relative to the 1st International Standard for Activated Factor IX (FIXa).

Lab	Type	Reagent	A			B			Ratio A/B
			GM IU/mL	% GCV	N	GM IU/mL	% GCV	N	
01	Chromogenic	Rossix	11.20	4.77	3	10.86	4.32	4	1.03
02	Chromogenic	Rossix	10.10	3.24	4	10.35	7.19	4	0.98
03	Fluorogenic	In- house	10.88	3.25	4	10.75	4.18	4	1.01
04	Chromogenic	Rossix	10.72	11.78	3	10.37	5.70	3	1.03
06	Chromogenic	Biophen	10.10	1.19	4	9.99	2.55	4	1.01
07	Chromogenic	Biophen	11.10	4.33	4	10.69	12.40	4	1.04
08a	Chromogenic	Biophen	8.72	17.50	4	9.87	3.39	3	0.88
08b	Chromogenic	Rossix	8.91	17.04	3	10.08	7.02	4	0.88
11	Chromogenic	In-house	9.82	-	2	9.26	-	2	1.06
14	Chromogenic	Rossix	10.72	4.51	4	10.57	6.85	4	1.01
15	Chromogenic	Rossix	10.79	6.82	4	10.65	8.11	4	1.01
17a	Chromogenic	Biophen	10.26	12.89	4	10.14	-	2	1.01
17b	Chromogenic	Rossix	10.49	-	1	10.55	12.21	3	0.99
18a	Chromogenic	In-house	10.63	8.40	4	11.23	10.22	4	0.95
18b	Chromogenic	Rossix	10.54	-	2	10.16	-	2	1.04
18c	Chromogenic	Biophen	11.25	3.46	4	11.01	2.60	4	1.02
	Overall	Incl all	10.36	7.68	16	10.42	4.81	16	0.99
		excl 08a 8b	10.60	4.13	14	10.48	4.84	14	1.01

Table 3: Purified reagent assays - Laboratory's geometric mean (GM) and Intra-laboratory variation expressed as % geometric coefficient of variation (%GCV) and overall GM and inter-laboratory GCV for samples A and B combined relative to the 1st International Standard for Activated Factor IX (FIXa)

			A & B		
Lab	Type	Reagent	GM IU/ml	% GCV	N
01	Chromogenic	Rossix	10.92	4.27	4
02	Chromogenic	Rossix	10.22	4.90	4
03	Fluorogenic	In- house	10.82	3.55	4
04	Chromogenic	Rossix	10.40	7.56	4
06	Chromogenic	Biophen	10.05	1.19	4
07	Chromogenic	Biophen	10.90	8.19	4
11	Chromogenic	IH-Pefachrome	9.43	4.99	3
14	Chromogenic	Rossix	10.65	4.46	4
15	Chromogenic	Rossix	10.72	6.97	4
17	Chromogenic	Biophen	9.98	9.60	4
17	Chromogenic	Rossix	10.67	11.16	3
18a	Chromogenic	IH	10.92	7.33	4
18b	Chromogenic	Rossix	10.35	-	2
18c	Chromogenic	Biophen	11.13	2.53	4
	Overall		10.48	4.68	14

Table 4: Clot based assays - Laboratory's geometric mean (GM) and Intra-laboratory variation expressed as % geometric coefficient of variation (%GCV) and overall GM and inter-laboratory GCV for samples A and B, the coded duplicates relative to the 1st International Standard for Activated Factor IX (FIXa). Due to the absence of valid estimates for sample A, Lab 12a results (for sample B) was excluded from calculation of overall GM for samples A and B combined

			A			B			Ratio A/B
Lab	Type	Reagent	GM IU/ml	%GCV	N	GM IU/ml	%GCV	N	
04	APTT	Actin-FS	11.67		2	11.51	8.55	4	1.01
05	APTT	CK-Prest 2	13.74	7.03	4	13.89	5.50	4	0.99
08	APTT	SynthAFax	10.98	2.76	4	10.95	5.66	4	1.00
10	APTT	APTT-SP	9.58	6.96	3	9.89	7.38	3	0.97
12a	APTT	Dapttin	-	-		12.36	-	1	-
16	APTT	APTT	10.59	2.11	4	10.36	1.37	4	1.02
19	APTT	PTTA	13.93	7.90	4	14.27	8.88	4	0.98
	Overall		11.65	15.76	6	11.80	15.19	7	0.99
09	NAPTT	CK-Prest 2	11.30	6.94	4	10.85	7.55	4	1.04
18	NAPTT	Rossix	13.17	4.00	4	13.23	5.07	4	1.00
	Overall		12.20		2	11.98		2	1.02

Table 5: Clot based assays - Laboratory's geometric mean (GM) and Intra-laboratory variation expressed as % geometric coefficient of variation (%GCV) and overall GM and inter-laboratory GCV for samples A and B combined relative to the 1st International Standard for Activated Factor IX (FIXa)

			A & B		
Lab	Type	Reagent	GM IU/ml	%GCV	N
04	APTT	Actin-FS	11.58	7.67	4
05	APTT	CK-Prest 2	13.82	6.07	4
08	APTT	SynthAFax	10.96	3.88	4
10	APTT	APTT-SP	9.73	6.81	3
16	APTT	APTT	10.47	0.89	4
19	APTT	PTTA	14.10	8.25	4
	Overall		11.67	16.20	6
09	NAPTT	CK-Prest 2	11.07	6.52	4
18	NAPTT	Rossix	13.20	2.79	4
	Overall		12.10	-	2

Table 6: Purified reagent assays - Laboratory's geometric mean (GM) and Intra-laboratory variation expressed as % geometric coefficient of variation (%GCV) and overall GM and inter-laboratory GCV for sample C relative to the 1st International Standard for Activated Factor IX (FIXa)

		C		
Lab	Reagent	GM IU/ml	%GCV	N
01	Rossix	0.015	2.35	4
02	Rossix	0.015	6.61	4
03	In-house	0.024	7.44	4
07	Biophen	0.015	-	1
08	Biophen	0.016	17.13	4
08	Rossix	0.010	-	2
14	Rossix	0.013	9.45	4
15	Rossix	0.012	1.16	4
18	Biophen	0.016	1.46	4
18	In-house	0.029	-	1
18	Rossix	0.013	-	2
	Overall	0.015	35.17	11

Table 7: Clot based assays - Laboratory's geometric mean (GM) and Intra-laboratory variation expressed as % geometric coefficient of variation (%GCV) and overall GM and inter-laboratory GCV for sample C relative to the 1st International Standard for Activated Factor IX (FIXa)

		C		
Lab	Reagent	GM IU/ml	%GCV	N
04	Actin-FS	2.59	6.52	3
16	APTT	2.96	5.22	4
05	CK-Prest 2	2.86	9.17	3
12a	Dapttin	5.29	-	1
12b	Siron LS	2.44	-	1
08	SynthAFax	3.72	3.44	3
	Overall	3.10	33.41	6
18	NAPTT	0.27	-	2

Table 8a:Thrombin generation test initiated by tissue factor – Lab 8 Laboratory's geometric mean (GM) and Intra-laboratory variation expressed as % geometric coefficient of variation (%GCV) relative to the 1st International Standard for Activated Factor IX (FIXa)

	A			B			C		
	GM IU/ml	%GCV	N	GM IU/ml	%GCV	N	GM IU/ml	%GCV	N
Clotting time	10.86	19.43	5	10.81	31.64	3	0.0126	-	1
Peak thrombin	10.78	22.66	4	12.45	19.37	2	0.0140	-	2
Time to Peak	10.69	21.94	4	11.63	8.34	2	0.0120	7.31	5

Table 8b: Thrombin generation test initiated by tissue factor and FXIa – Lab 8 Laboratory's geometric mean (GM) and Intra-laboratory variation expressed as % geometric coefficient of variation (%GCV) relative to the 1st International Standard for Activated Factor IX (FIXa)

	A			B			C		
	GM IU/ml	%GCV	N	GM IU/ml	%GCV	N	GM IU/ml	%GCV	N
Clotting time	10.27	15.62	4	9.30	3.81	4	0.105	-	2
Peak thrombin	10.84	10.68	4	9.93	4.12	5	0.200	14.02	5
Time to Peak	11.11	5.82	4	10.93	12.47	3	0.163	3.74	3

Table 9: Comparison of overall potency estimates by different assay methods for samples A and B combined and sample C

	A and B			C		
	Potencies IU/mL	% GCV	n	Potencies IU/mL	% GCV	n
Purified reagent assays	10.48	4.68	14	0.015	35.17	11
APTT	11.67	16.20	6	3.10	33.41	6
NAPTT	12.10	-	2	0.27	-	1

Table 10a: Accelerated degradation study on candidate, 14/316. Four time-points, results fitted to Arrhenius Equation for prediction of loss of FIXa function activity per year.

FIXa functional activity by purified reagent assay		
Storage temperature (°C)	Predicted loss per year (%) (relative to -150)	95% upper confidence limit (% loss)
-150	0	0
-70	0	0
-20	0	0
4	0.01	0.015
20	0.366	0.461
37	10.432	11.62

Table 10b: On-bench stability of candidate, 14/316. An ampoule of the candidate preparation was reconstituted and stored on melting ice. An aliqote was assayed against a freshly reconstituted ampoule of 14/316 using a FIXa functional chromogenic assay, with the activity of the fresh ampoule assumed to be 100%.

Time on melting ice	% FIXa functional activity (95% confidence limit)
0 h	97.44 (94.85-100.11)
1 h	97.31 (93.97-100.78)
2 h	97.85 (SW) (92.75-103.22)
3 h	99.74 (96.74-102.84)
4 h	97.29 (92.87-101.91)

Figure 1: Histogram showing estimated potency of samples A and B by purified reagent assays, relative to the 1st International Standard for Activated Factor IX (FIXa). Each box denotes overall geometric mean potency (GM) from one laboratory

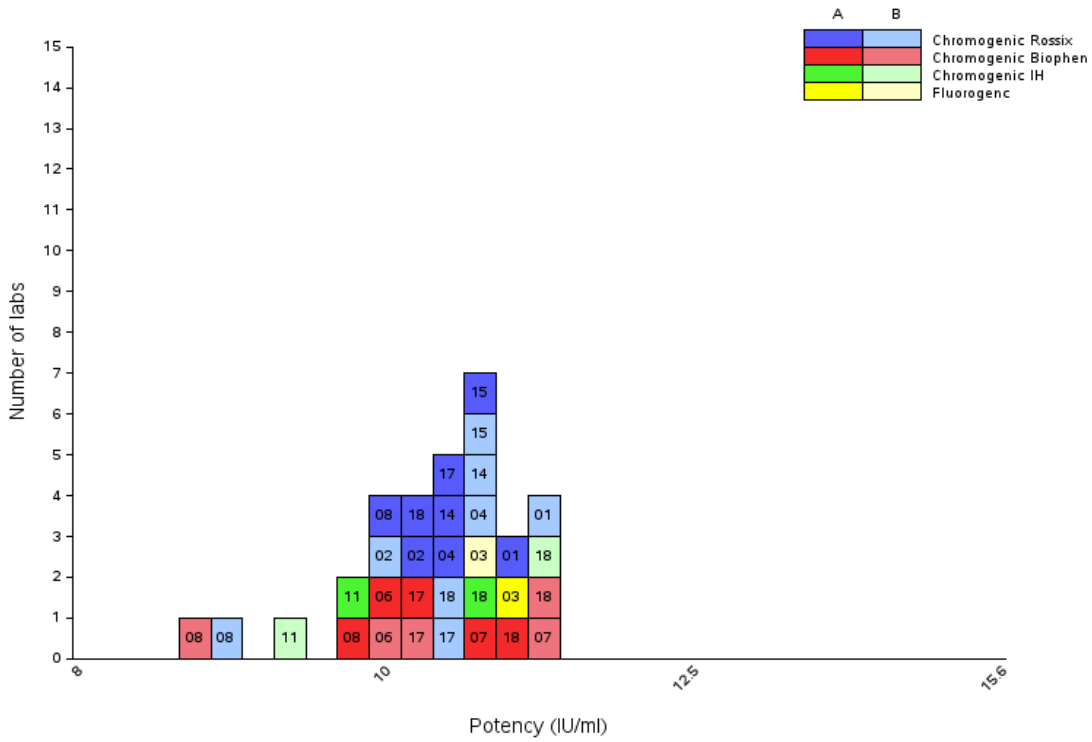


Fig 2: Histogram showing estimated potency of samples A and B by clot based assays, relative to the 1st International Standard for Activated Factor IX (FIXa). Each box denotes overall geometric mean potency (GM) from one laboratory

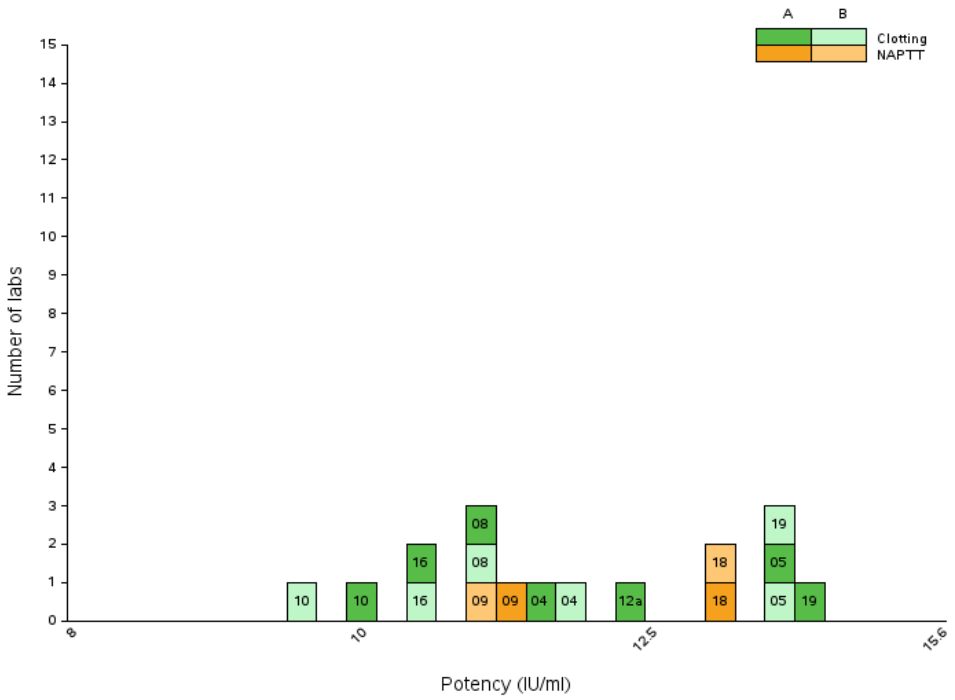
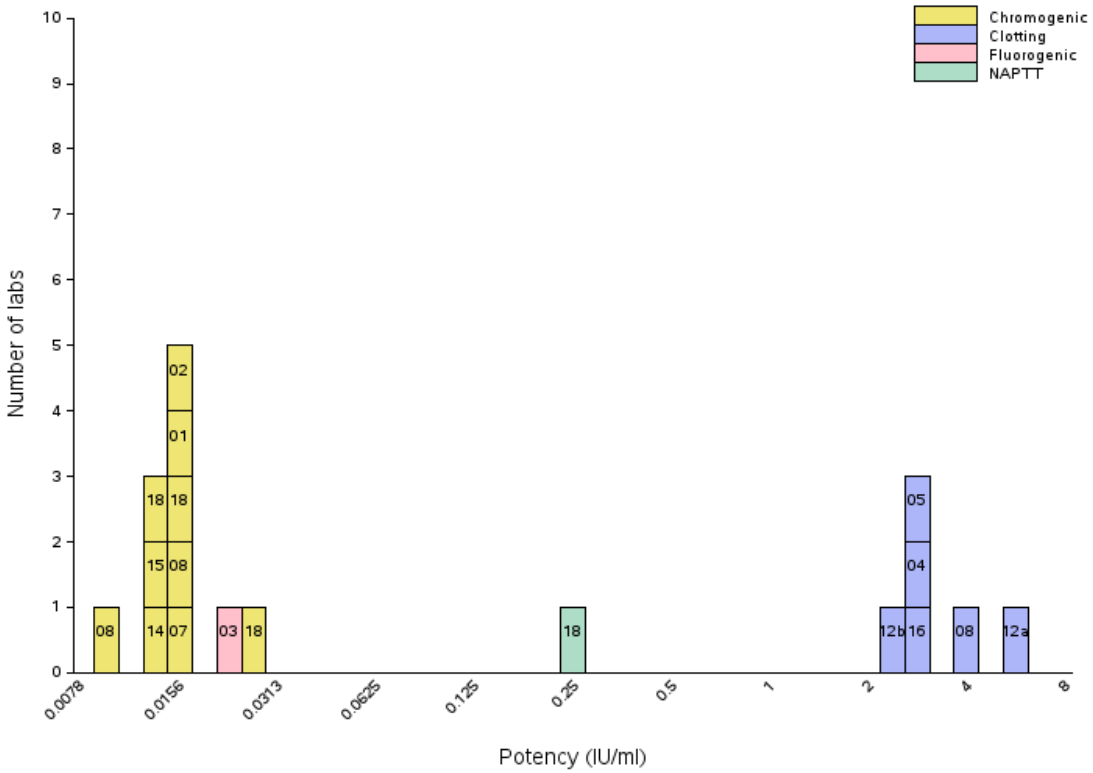


Fig 3: Histogram showing estimated potency of sample C relative to the 1st International Standard for Activated Factor IX (FIXa). Each box denotes overall geometric mean potency (GM) from one laboratory



APPENDIX I: List of Participants

Stephanie Eichmeir, AGES MEA, Vienna, Austria

Karin Fuchs, Biotest AG, Dreieich, Germany

Martina Treutlein and Marika Midon, CSL Behring GmbH, Marburg, Germany

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Herbert Gritsch, Shire, Vienna, Austria

Francois Hemery, LFB Biomedicaments, Lille, France

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Lisa Reid-Fifoot, Precision Biologic Inc, Dartmouth, NS, Canada

Per Rosén, Rossix AB, Molndal, Sweden

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Helen Wilmot and Graham Roberts, NIBSC, Potters Bar, UK

Nikolaus Binder, Technoclone Herstellung von Diagnostik- a und Arzneimitteln GmbH, Vienna, Austria

Appendix II: Study protocol

VALUE ASSIGNMENT OF THE PROPOSED 2nd INTERNATIONAL STANDARD FOR ACTIVATED FACTOR IX (FIXa) (14/316).

CS545 STUDY PROTOCOL

1 SAMPLES FOR ASSAY

CODE	PREPARATION
S	1st International Standard for Activated Factor IX (FIXa) (97/562), containing 11 IU FIXa per ampoule
A	Candidate A, containing approximately 10 units FIXa per ampoule
B	Candidate B, containing approximately 10 units FIXa per ampoule
C	Sample C, a FIX preparation containing approximately 10 IU/ml of FIX and approximately 0.01 IU/ml of FIXa

Note: the unit of FIXa as defined by the International Standard is not the same as one unit of purified factor IX when fully activated.

2 STORAGE AND RECONSTITUTION OF AMPOULES S, A AND B

Store all unopened ampoules at -20°C or below. For reconstitution, ampoules should first be allowed to warm to room temperature and subsequently reconstituted by the addition of 1.0 ml of distilled water (see previously supplied instructions for use on how to open the ampoule). Allow the contents to solubilise for 5-10 minutes at room temperature with gentle mixing, transfer contents to a plastic tube and store on melting ice.

3 OUTLINE OF STUDY

A total of four assays should be carried out over 4 separate days, using fresh ampoules of S, A, B and C in each assay. Assays should be begun as soon as possible after reconstitution and completed within 2 hours of reconstitution. Dilutions should be adjusted as necessary after assay 1 if the dose-response is not linear or the data for A, B or C do not overlap that of sample S.

4 ASSAY DESIGN – chromogenic assay

If the chromogenic assay is automated on a coagulometer, please use the balanced testing order suggested in table 1.

TABLE 1: Suggested balanced order for automated chromogenic kits

Assay 1	**S**	**A**	**B**	**C**	**C'**	**B'**	**A'**	**S'**
Assay 2	**A**	**B**	**C**	**S**	**S'**	**C'**	**B'**	**A'**
Assay 3	**B**	**C**	**S**	**A**	**A'**	**S'**	**C'**	**B'**
Assay 4	**C**	**S**	**A**	**B**	**B'**	**A'**	**S'**	**C'**

where each letter refers to a set of different dilutions (please use four dilutions if possible, for example 1/2, 1/4, 1/8, 1/16) and **A, A'** and **S, S'** etc. refer to separate sets of dilutions (replicates) made independently from the same ampoule. The range of dilutions should be chosen to lie on the most linear portion of the dose-response relationship. The same range of dilutions should be used for all materials (**S, A, B, C**), if necessary by performing a pre-dilution step. The assays should be completed within **two hours** of reconstitution.

If the method is being performed manually on a microtitre plate, please use a balanced design such as suggested below:

Assay 1:

		S1a	S1b	B1a	B1b						
		S2a	S2b	B2a	B2b						
		S3a	S3b	B3a	B3b						
		S4a	S4b	B4a	B4b						
		A1a	A1b	C1a	C1b						
		A2a	A2b	C2a	C2b						
		A3a	A3b	C3a	C3b						
		A4a	A4b	C4a	C4b						

Assay 2:

		A1a	A1b	C1a	C1b						
		A2a	A2b	C2a	C2b						
		A3a	A3b	C3a	C3b						
		A4a	A4b	C4a	C4b						
		B1a	B1b	S1a	S1b						
		B2a	B2b	S2a	S2b						
		B3a	B3b	S3a	S3b						
		B4a	B4b	S4a	S4b						

Assay 3:

		B1a	B1b	S1a	S1b						
		B2a	B2b	S2a	S2b						
		B3a	B3b	S3a	S3b						
		B4a	B4b	S4a	S4b						
		C1a	C1b	A1a	A1b						
		C2a	C2b	A2a	A2b						
		C3a	C3b	A3a	A3b						
		C4a	C4b	A4a	A4b						

Assay 4:

		C1a	C1b	A1a	A1b						
		C2a	C2b	A2a	A2b						

		C3a	C3b	A3a	A3b						
		C4a	C4b	A4a	A4b						
		S1a	S1b	B1a	B1b						
		S2a	S2b	B2a	B2b						
		S3a	S3b	B3a	B3b						
		S4a	S4b	B4a	B4b						

Each letter (S,A,B,C) refers to the sample code; each number refers to the dilution (e.g. 1=1/2; 2=1/4 and so on) and a,b refer to separate sets of dilutions (replicates) made independently from the same ampoule (e.g. S1a = sample S diluted 1/2; S1b = sample S dilution 1/2 (independent dilution to S1a)). The range of dilutions should be chosen to lie on the most linear portion of the dose-response relationship. The same range of dilutions should be used for all materials (**S, A, B, C**), if necessary by performing a pre-dilution step. The assays should be completed within **two hours** of reconstitution

4 ASSAY DESIGN – clotting assay

A balanced order of testing should be followed, for example:

Assay 1	**S**	**A**	**B**	**C**	**C'**	**B'**	**A'**	**S'**
Assay 2	**A**	**B**	**C**	**S**	**S'**	**C'**	**B'**	**A'**
Assay 3	**B**	**C**	**S**	**A**	**A'**	**S'**	**C'**	**B'**
Assay 4	**C**	**S**	**A**	**B**	**B'**	**A'**	**S'**	**C'**

where each letter refers to a set of different dilutions (please use four dilutions if possible, for example 1/10, 1/30, 1/50, 1/100) and **A, A'** and **S, S'** etc. refer to separate sets of dilutions (replicates) made independently from the same ampoule. The range of dilutions should be chosen to lie on the most linear portion of the dose-response relationship. The same range of dilutions should be used for all materials (**S, A, B, C**), if necessary by performing a pre-dilution step. The assays should be completed within **two hours** of reconstitution.

Please note that the APTT clotting assay detects both FIX and FIXa.

If you are using two different APTT reagents for the study, 4 separate assays should be performed for each reagent (total 8). You may, however, use the same set of reconstituted samples for the second APTT reagent (therefore performing 2 assays on each day), but please prepare fresh dilutions for each assay and complete the assays within 2 hours of reconstitution.

5 RESULTS

Raw data (e.g. absorbance or clotting times) should be recorded on the results sheets provided. You are also invited to calculate the relative potencies of A, B and C vs S from your own assay results using the assigned potencies of S given in section 1. Please return your raw data and calculated potency estimates by **12th September 2016** to elaine.gray@nibsc.org

Appendix III A: Individual assay results for chromogenic, fluorogenic, APTT and NAPTT - Laboratory's reported and NIBSC estimated potencies in IU/mL relative to 1st International Standard for Activated Factor IX (FIXa). NL= non-linear; NP= non-parallel; OoR= test responses out of standard dose response range; Std NL = Standard dose response non-linear; NA= no returned data; InD=insufficient doses

Lab / Method	Sample	Lab's reported results				NIBSC estimated potencies			
		Assay 1	**Assay 2**	**Assay 3**	**Assay 4**	**Assay 1**	**Assay 2**	**Assay 3**	**Assay 4**
1 / Chromo / Rossix	A	10.70	11.11	10.32	11.52	10.7	11.2	NL	11.7
	B	11.23	10.39	10.55	11.29	11.2	10.6	10.4	11.3
	C	0.01542	0.01571	0.01524	0.01570	0.01506	0.01549	0.01492	0.01568
2 / Chromo / Rossix	A	10.64	10.45	9.95	9.96	10.5	10.3	9.8	9.9
	B	10.88	11.25	10.34	9.58	10.7	11.1	10.2	9.5
	C	0.0161	0.0158	0.0146	0.0140	0.016	0.016	0.015	0.014
3 / Fluoro	A	10.45	11.12	10.70	11.06	10.58	11.35	10.64	10.97
	B	10.68	11.04	10.43	10.86	10.74	11.29	10.22	10.79
	C	0.0240	0.0254	0.0226	0.0261	0.0240	0.0258	0.0222	0.0258
4a / Clot / Actin-FS	A	12.9	10.5	11.7	10.7	12.63	NL	NL	10.78
	B	13.2	11.4	11.3	10.3	12.53	11.90	11.40	10.30
	C	NT	NT	NT	NT	NP	2.55	2.77	2.45
4b / Chromo / Rossix	A	10.30	10.30	11.50	10.50	10.08	10.02	12.19	NL
	B	11.40	10.70	10.50	10.10	NL	10.21	11.02	9.90
	C	-	-	-	-	NL	OoR	NP	OoR
5 / Clot / CK-Prest	A	14.9	13.4	12.4	13.9	14.881	13.720	12.608	13.865
	B	14.7	13.2	13.1	13.8	15.020	13.506	13.346	13.758
	C	*2.9*	*3.1*	*2.6*	*2.8*	2.96	3.05	2.59	NP
6 / Chromo / Hyphen	A	10.20	10.06	10.33	9.94	10.3	10.1	10.1	10.0
	B	9.92	9.94	9.95	10.28	10.03	9.93	9.71	10.31
	C	0.010	NA	0.014	0.013	NP		NP	NP
7 / Chromo / Hyphen	A	11.44	11.05	10.86	10.63	11.31	11.38	10.42	11.33
	B	10.74	11.39	9.81	10.04	11.08	12.02	9.10	10.78
	C	0.0121	0.0114	0.0117	0.0113	NP	NP	0.0146	NP
8a / Chromo / Hyphen	A	7.71	8.47	8.07	10.85	7.71	8.25	8.22	11.05
	B	9.55	9.69	10.01	9.58	9.67	NL	10.26	9.70
	C	0.01618	0.01533	0.01197	0.01816	0.01706	0.01562	0.01301	0.01888
8b / Chromo / Rossix	A	8.44	9.99	8.03	10.76	8.41	NP	7.90	10.65
	B	9.51	11.26	9.45	10.13	9.50	11.01	9.62	10.27
	C	0.010	0.005	0.009	0.012	NP	NL	0.009	0.012
8c / Clot / Synthafax	A	11.03	11.01	11.79	10.77	11.02	11.02	11.30	10.58
	B	11.52	10.62	11.51	11.02	11.51	10.43	11.46	10.45

	C	3.73	3.46	3.62	4.06	3.72	NL	3.60	3.85
9 / Clot / CK-Prest	A	10.67	12.22	10.97	10.47	10.93	12.46	11.18	10.72
	B	10.52	11.09	11.38	9.71	10.76	11.35	11.56	9.83
	C	0.0043	0.0046	0.0042	0.0039	OoR	OoR	OoR	OoR
10 / Clot / APTT-SP	A	None provided				Std NL	9.53	8.99	10.28
	B					Std NL	10.32	9.11	10.28
	C					Std NL	NP	NP	NP
11 Chromo / IH-PefaChromoome	A	11.03	9.89	9.37	9.80	Std NL	10.13	NP	9.52
	B	10.70	9.54	8.57	8.47	Std NL	9.58	8.95	NP
	C	< 0,11	< 0,11	< 0,11	< 0,11	OoR	OoR	OoR	OoR
12a / Clot / Diapttin	A	None provided				NP	NP	NP	NP
	B					NP	NP	NP	12.36
	C					NL	NP	5.29	NL
12b / Clot / Siron LS	A	None provided				Std NL	NL	Std NL	NP
	B					Std NL	NL	Std NL	NP
	C					Std NL	2.44	Std NL	NP
13a / Chromo / Hyphen	A	None provided				Std NL	Std NL	Std NL	Std NL
	B					Std NL	Std NL	Std NL	Std NL
	C					No Response	No Response	No Response	No Response
13b / Clot / APTT-SP	A	None provided				Std NL	Std NL	Std NL	-
	B					Std NL	Std NL	Std NL	-
	C					No Response	No Response	No Response	-
14 / Chromo / Rossix	A	11.00	10.50	10.30	11.40	10.85	10.36	10.36	11.36
	B	9.90	10.30	10.90	11.40	9.69	10.44	10.95	11.28
	C	0.01100	0.01200	0.01300	0.01400	0.01152	0.01225	0.01399	0.01358
15 / Chromo / Rossix	A	11.67	10.22	10.83	10.58	11.73	10.12	10.99	10.37
	B	11.83	10.51	10.15	10.37	11.95	10.42	10.09	10.24
	C	0.01200	0.01300	0.01200	0.01300	0.01219	0.01242	0.01242	0.01216
16 / Clot / APTT	A	10.551	10.817	10.586	10.191	10.41	10.91	10.58	10.47
	B	10.113	10.504	10.135	10.282	10.28	10.20	10.50	10.45
	C	2.792	2.947	2.874	2.841	2.78	3.15	2.97	2.94
17a / Chromo / Hyphen	A	None provided				9.69	11.35	8.89	11.35
	B					NL	9.83	NL	10.46
	C					OoR	OoR	OoR	OoR
17b / Chromo / Rossix	A	None provided				10.49	NL	Std NL	NL
	B					9.81	12.05	Std NL	9.94
	C					OoR	OoR	OoR	OoR
18a / Chromo / In-house	A	9.85	10.91	11.61	10.11	9.82	10.97	11.72	10.10
	B	10.06	10.73	11.61	12.47	10.04	10.77	11.72	12.55
	C	0.0150	0.0160	0.0230	0.0270	NP	NP	NP	0.0288
18b / Chromo /	A	10.32	9.70	10.70	14.72	10.3	Std NL	10.8	Std NL

Rossix	B	10.04	10.17	10.57	10.19	10.0	Std NL	10.3	Std NL
	C	0.01	0.01	0.01	0.02	0.0	Std NL	0.0	Std NL
18c / Chromo / Hyphen	A	11.15	11.51	10.37	11.86	11.39	11.29	10.72	11.61
	B	10.71	10.64	10.79	11.49	10.88	10.76	10.99	11.42
	C	0.0160	0.0160	0.0160	0.0170	0.0162	0.0162	0.0162	0.0167
18d / Clot / NAPTT	A	12.93	13.06	14.40	11.96	12.93	13.06	13.94	12.77
	B	12.76	14.21	13.16	12.71	12.76	14.21	12.84	13.15
	C	0.2853	0.2499	0.1817	0.1541	0.2853	0.2499	NP	NL
19 / Clot / PTTA	A	13.20	13.00	13.80	15.50	13.26	13.29	13.72	15.57
	B	13.80	13.40	14.10	15.30	13.67	13.10	14.54	15.93
	C	1.60	/	1.30	1.40	InD	InD	InD	InD

Appendix III B: Lab 8, Individual assay results for thrombin generation test based methods**.**
- Laboratory's reported and NIBSC estimated potencies n IU/mL relative to 1st International Standard for Activated Factor IX (FIXa). NL= non-linear; NP= non-parallel; OoR= test responses out of standard dose response range; Std NL = Standard dose response non-linear; NA= no returned data.

Reagents used in TGT:

Tissue Factor – Recombiplastin (Werfen), final concentration ~0.35 pM or ~2.63 U/mL in units of NIBSC TF TGT reagent 14/238
FXIa – frozen aliquots of WHO Interantioanl Reference Reagent for FXIa, 11/236 final concentration ~10 pM or ~17.54 mIU/mL in units of NIBSC 11/236
PC:PS vesicles:Rossix TGT final concentration 4 uM
Plasma volume: 50 % vol/vol
Substrate: 800 uM ZGGR-AMC (Bachem)
CaCl2: 10 uM

- Note that TG experiments were initiated by adding [plasma/lipids/TF/FIXa/FIX] to a mixture of [Calcium/Substrate/FXIa]

Lab 8 Tissue factor initiated, microplate clot time assay

	Lab's reported results					NIBSC estimated potencies				
	Assay 1	**Assay 2**	**Assay 3**	**Assay 4**	**Assay 5**	**Assay 1**	**Assay 2**	**Assay 3**	**Assay 4**	**Assay 5**
A	10.8	12.3	15.1	9.4	9.6	11.16	12.16	13.48	8.79	9.38
B	8.6	13.7	13.9	9.1	11.2	8.15	14.11	NL	NL	10.99
C	0.012	0.017	0.022	0.014	0.014	NL	0.0126	OoR	NL	NP

Lab 8 Tissue factor initiated, thrombin peak height

	Lab's reported results					NIBSC estimated potencies				
	Assay 1	**Assay 2**	**Assay 3**	**Assay 4**	**Assay 5**	**Assay 1**	**Assay 2**	**Assay 3**	**Assay 4**	**Assay 5**
A	10.2	12.2	11.2	12.1	10.9	Std NL	11.76	11.49	10.88	10.36
B	9.6	12.4	11.2	9.5	10.0	Std NL	12.30	10.92	NL	9.73
C	0.010	0.013	0.012	0.011	0.012	Std NL	0.0153	NL	NP	0.0139

Lab 8 Tissue factor initiated, time to peak										
	Lab's reported results					NIBSC estimated potencies				
	Assay 1	**Assay 2**	**Assay 3**	**Assay 4**	**Assay 5**	**Assay 1**	**Assay 2**	**Assay 3**	**Assay 4**	**Assay 5**
A	10.6	10.0	11.8	10.9	9.8	10.58	9.88	11.64	10.84	9.76
B	11.6	9.8	10.8	11.1	10.3	11.61	9.79	10.66	11.03	10.26
C	0.014	0.012	0.014	0.012	0.013	0.0131	0.0129	0.0130	0.0114	0.0138

Lab 8 Tissue factor-XIa initiated, microplate clot time assay										
	Lab's reported results					NIBSC estimated potencies				
	Assay 1	**Assay 2**	**Assay 3**	**Assay 4**	**Assay 5**	**Assay 1**	**Assay 2**	**Assay 3**	**Assay 4**	**Assay 5**
A	15.8	8.9	15.2	7.2	7.2	NP	NP	Std NL	6.57	NL
B	10.1	10.4	11.5	9.3	9.5	9.21	9.82	Std NL	9.20	9.00
C	0.16	0.09	0.25	0.11	0.09	0.1527	NL	Std NL	NL	0.0725

Lab 8 Tissue factor-XIa initiated, thrombin peak height										
	Lab's reported results					NIBSC estimated potencies				
	Assay 1	**Assay 2**	**Assay 3**	**Assay 4**	**Assay 5**	**Assay 1**	**Assay 2**	**Assay 3**	**Assay 4**	**Assay 5**
A	10.0	12.2	9.3	10.5	11.2	9.96	10.93	9.26	10.41	11.11
B	10.4	10.5	9.9	9.4	9.8	10.44	10.17	9.85	9.38	9.83
C	0.20	0.19	0.18	0.22	0.23	0.2006	0.1843	0.1683	0.2269	0.2274

Lab 8 Tissue factor-XIa initiated, time to peak										
	Lab's reported results					NIBSC estimated potencies				
	Assay 1	**Assay 2**	**Assay 3**	**Assay 4**	**Assay 5**	**Assay 1**	**Assay 2**	**Assay 3**	**Assay 4**	**Assay 5**
A	10.8	10.8	10.0	10.0	9.9	Std NL	10.43	NP	10.42	10.42
B	10.3	10.8	10.0	9.4	10.2	Std NL	10.43	10.00	9.60	10.00
C	0.20	0.17	0.18	0.18	0.16	Std NL	0.1578	NL	0.1630	0.1698

Appendix IV: Draft Instruction for Use

Medicines & Healthcare products Regulatory Agency

NIBSC Confidence in Biological Medicines

WHO International Standard
2nd International Standard for FIXa
NIBSC code: 14/316
Instructions for use
(Version 1.00, Dated)

1. INTENDED USE

The 2nd International Standard for Activated Factor IX (FIXa), Human, coded14/316 consists of ampoules containing aliquots of a freeze-dried purified human FIXa prepared from activated recombinant human Factor IX. This preparation was established as the 2nd International Standard for Activated Factor IX, Human, by the Expert Committee on Biological Standardisation of the World Health Organisation in October 2017. The intended use of this standard is for measurement of FIXa.

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 of the 2nd International Standard for FIXa was calibrated by 19 laboratories from 9 different countries against the 1st International Standard for FIXa, 97/562 by purified reagent functional activity methods specific for FIXa. The assigned potency of this preparation is

10.5 IU/ampoule.

The unit of FIXa as defined by this standard is not identical to a unit of purified Factor IX when fully activated.

4. CONTENTS

Country of origin of biological material: USA.
The bulk starting material for 14/316 was prepared by FXIa activation of human recombinant FIX. The purity of the FIXa was assessed and confirmed by PAGE with silver staining. The estimated specific activity of the bulk was 612 IU/mg. Sixy-two ml of the frozen bulk was thawed at 37°C diluted in buffer (0.05MTris, 0.15M NaCl, 5 mg/ml trehalose, 1.25% human albumin, pH 7.4) to approximately 10 IU/ml. The solution was distributed into approximately 18,000 ampoules at 4°C and freeze-dried. The mean weight of the liquid content 1.0083g (n = 410). As WHO International Standard, there is no uncertainty associated with the assigned value of 14/316. Where required the uncertainty of the ampoule content is taken as the coefficient of variation of the fill which has been estimated to be 0.169%.

5. STORAGE

PUnopened ampoules should be stored in the dark at or below -20°C. Allow ampoules to warm to room temperature before opening and reconstitution.
Please note: because of the inherent stability of lyophilized material, NIBSC may ship these materials at ambient temperature.

6. DIRECTIONS FOR OPENING

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

7. USE OF MATERIAL

No attempt should be made to weigh out any portion of the freeze-dried material prior to reconstitution
Open ampoule, taking acre to ensure that all material is in the lower part and reconstitute with 1.0 ml of distilled water. The reconstituted Standard should be used as soon as possible. On-bench stability study in one laboratory suggests the reconstituted standard would be stable up to 4 hours when stored on melting ice.

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.t is the policy of WHO not to assign an expiry date to their international reference materials. They remain valid with the assigned potency and status until withdrawn or amended.

Accelerated degradation study, which involves potency estimation of ampoules stored at elevated temperatures relative to ampoules stored at below -150°C, of 14/316 showed no predicted loss of FXII activity when the preparation is stored at -20°C or below. The predicted loss for Standard stored at +20°C was 0.4% per year and this supports shipment at ambient temperature.The accelerated degradation study and real time monitoring will continue for the lifetime of the standard

9. REFERENCES

N/A

10. ACKNOWLEDGEMENTS

Pfizer Inc (Andover, Massachusetts, USA) for kind donation of the bulk FIXa. the participants of the international collabortive study

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

National Institute for Biological Standards and Control,
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WHO International Laboratory for Biological Standards,
UK Official Medicines Control Laboratory

World Health Organization

Medicines & Healthcare products Regulatory Agency

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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 solid		Corrosive:	No
Stable:	Yes	Oxidising:	No
Hygroscopic:	Yes	Irritant:	Yes
Flammable:	No	Handling:	See caution, Section 2
Other (specify):	contains recombinant and human plasma derived material		

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

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

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

15. LIABILITY AND LOSS
In the event that this document is translated into another language, the English language version shall prevail in the event of any inconsistencies between the documents.
Unless expressly stated otherwise by NIBSC, NIBSC's Standard Terms and Conditions for the Supply of Materials (available at http://www.nibsc.org/About_Us/Terms_and_Conditions.aspx or upon request by the Recipient) ("Conditions") apply to the exclusion of all other terms and are hereby incorporated into this document by reference. The Recipient's attention is drawn in particular to the provisions of clause 11 of the Conditions.

16. INFORMATION FOR CUSTOMS USE ONLY

Country of origin for customs purposes*: United Kingdom * Defined as the country where the goods have been produced and/or sufficiently processed to be classed as originating from the country of supply, for example a change of state such as freeze-drying.
Net weight: 0.03g
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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World Health Organization