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Collaborative study for the establishment of a replacement WHO International Standard for tetanus immunoglobulin (human) and assessment of commutability

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Summary

This report describes the outcome of a project to develop a replacement World Health Organization (WHO) International Standard (IS) and European Pharmacopoeia (Ph. Eur.) Biological Reference Preparation (BRP) for tetanus immunoglobulin human (TIg). Bulk TIg was kindly provided by a European manufacturer and was used to prepare the candidate standard. The candidate standard was freeze-dried and calibrated in an international collaborative study coordinated by NIBSC and EDQM. The results of this study show that there was good agreement between laboratories for the potency estimates obtained for the candidate standard relative to the current WHO IS/Ph. Eur. BRP. The study also demonstrated that the candidate standard is suitable for use in Pharmacopoeia assays that are used for potency testing of TIg products and there was good agreement in the potency estimates obtained using the different assay methods included in the study. Accelerated degradation studies performed at NIBSC over a period of 4 years suggest that the freeze-dried candidate standard will be very stable. Results of a commutability study performed at NIBSC suggest that the candidate standard is commutable with patient samples across a range of tetanus immunoassays. It is proposed that the candidate standard is established as the 2nd WHO International Standard for Tetanus Immunoglobulin Human with an assigned value of 45 IU/ampoule.

Introduction

The International Unit (IU) for tetanus antitoxin is defined by the WHO IS (coded TE-3). TE-3, prepared from a batch of human TIg, was established by WHO in 1992 as a replacement for a previous IS of equine origin [1]. The same preparation (TE-3) was also established by the European Pharmacopoeia Commission in 1993 as the Ph. Eur. Biological Reference Preparation (BRP) for Human Tetanus Immunoglobulin, Batch 1 [1]. TE-3 is used for calibration (tetanus potency) of therapeutic preparations of TIg and is the Pharmacopoeia reference preparation for the Ph. Eur. monograph *Human tetanus immunoglobulin (0398)* for the test for toxin neutralizing capacity in mice and for potency measurement by immunoassay [2]. The WHO IS is also used for calibration of serological assays used to measure anti-tetanus antibody levels in human serum and there are a number of commercial tetanus ELISA kits available that report results in IU. Due to low stocks of TE-3 a project was initiated to prepare and establish a replacement WHO IS and Ph. Eur. BRP. This report details the development and characterisation of the candidate replacement standard (coded 13/240) performed at NIBSC and the results of an international collaborative study jointly coordinated by NIBSC and EDQM. Because the IS is also used for calibration of immunoassays used to measure anti-tetanus antibody levels in human serum, a separate study was also performed to assess commutability. The commutability study was performed at NIBSC (not as part of the international collaborative study) and is described in an appendix to this report.

Bulk material and processing

Bulk liquid TIg was kindly donated by the Institute of Immunology in Zagreb (Croatia). The bulk material was supplied with a certificate of analysis (CoA) and safety data sheet. The CoA confirmed that all quality requirements had been met and that tests for relevant viral markers were negative. The characteristics of the source material are shown in Table 1. The bulk TIg had a protein content of 154.5 g/L and an estimated tetanus potency of 148.7 IU/mL.

A trial fill was performed at NIBSC using a small aliquot of the bulk TIg without any additional formulation or dilution. Approximately 55 mL was removed under aseptic conditions in a class II microbiological safety cabinet (MSC) and was distributed into ampoules (1 mL per ampoule) and freeze-dried. The freeze-dried product was of good appearance but did not reconstitute readily – after addition of 1 mL of sterile water or phosphate buffered saline (PBS) the ampoule contents were not completely dissolved even after 1h. Results from size-exclusion (SE)-HPLC analysis showed that the molecular size distribution was good and that the solubility problem was not caused by extensive aggregation on freeze-drying. The solubility problem was thought to be due to the high protein content (~15%) of the bulk TIg. Further process development studies were therefore performed.

A second trial fill compared two different formulations that were diluted 1/3 (to give an estimated tetanus potency of 50 IU/mL and protein content of ~5%). Formulation A was diluted 1/3 in sterile water and formulation B was diluted 1/3 in sterile water containing 21.2 g/L glycine (to maintain the glycine concentration in the original bulk material). Formulations were prepared under aseptic conditions in a class II MSC. After freeze-drying, both formulation A and B were of good appearance and had comparable molecular size distributions. Formulation B (with glycine) displayed rapid reconstitution after addition of 1 mL sterile water while formulation A (no added glycine) was slower to reconstitute (approx. 5 min). Tetanus potency was determined by ELISA and, in comparison to the relevant formulated liquid bulk, formulation A lost approximately 22% of activity on freeze-drying and formulation B lost approximately 5% of activity on freeze-drying, suggesting that maintaining the glycine concentration during freeze-drying helped to retain activity. The potency of formulation B was also confirmed by mouse bioassay and there was no difference in potency for the formulated liquid and freeze-dried preparation where the potency of both preparations (i.e. liquid and freeze-dried) was close to the expected value of 50 IU/mL.

A short accelerated degradation stability study was also performed using formulations A and B. Tetanus potency determined by ELISA confirmed that there was no loss of activity for either formulation A or B (at temperatures up to 45°C for 8 weeks) when expressed relative to ampoules of the same formulation held at the recommended storage temperature of -20°C. Based on the results of these process development studies a decision was made to proceed to definitive fill using formulation B (bulk TIg diluted 1/3 in sterile water maintaining the glycine content at 21.2 g/L).

Definitive fill

Bulk TIg batch 1049 (6 L) was mixed with 12 L of sterile water containing 21.2 g/L glycine. The candidate material was prepared in a class II MSC under aseptic conditions. Filling (1 mL fill into 5 mL ampoules) was performed on 13th March 2014 within NIBSC's Standards Processing Division using a Bausch and Strobel Filling Machine (AFV5090). The material was stirred constantly during filling and the temperature was maintained between +2-8°C. The filled ampoules were freeze-dried using a 4-day cycle, with primary drying at -35°C and secondary drying at +30°C in a Serial CS100 freeze-dryer (Serial, Le Coudray Saint Germer, France). Ampoules were back-filled to atmospheric pressure using boil-off gas from high purity (99.99%) liquid nitrogen and flame sealed to give homogeneous glass containers. The finished product was coded 13/240. Specifications were met for the precision of fill, residual moisture content by coulometric Karl Fischer titration (Mitsubishi CA-100, A1 Envirosciences Ltd. Blyth, UK) and mean residual oxygen headspace by non-invasive frequency modulated spectroscopy (FMS 760, Lighthouse Instruments, Charlottesville, USA). The results are

summarised in Table 2. Microbiological testing returned bacterial and mould/yeast colony counts of 0 Cfu/mL in pre- and post-filled samples.

Post-fill and freeze-drying characterisation tests for 13/240 performed at NIBSC included tests for appearance and reconstitution, tetanus potency (ELISA and mouse bioassay) and molecular size distribution by SE-HPLC. The freeze-dried product 13/240 formed a robust cake and reconstituted readily in sterile water or PBS. Tetanus potency by ELISA was estimated to be 50.4 (49.2 – 51.6) IU/mL compared to 53.8 (49.2 – 58.9) IU/mL for the liquid bulk suggesting ~6% loss of activity on freeze-drying. Tetanus potency by mouse bioassay was estimated to be 46 IU/mL (geometric mean of two independent assays) compared to 52 IU/mL for the liquid bulk (geometric mean of two independent assays) suggesting ~12% loss of biological activity on freeze-drying. A total of 9 ampoules taken from across the production run were tested for tetanus potency by ELISA for assessing homogeneity across the batch. There was no significant difference between them (and the differences in measured potency between ampoules is not greater than differences in potency for the same ampoule tested on different ELISA plates), suggesting that the homogeneity of the batch is good. Analysis of molecular size distribution by SE-HPLC confirmed that 13/240 had an acceptable profile, comparable to that of the liquid bulk material, with <3% polymer/aggregates. The post fill results for 13/240 are summarised in Table 3 and Table 4.

A total of 18,034 ampoules of 13/240 were filled at NIBSC. Following use of material for post-fill characterization studies (including stability studies), the international collaborative study and transfer of a portion of the batch to EDQM there are 16,000 ampoules offered to WHO for establishment of the replacement International Standard. NIBSC will act as the custodian of 13/240 and ampoules will be held at -20 °C in the dark at NIBSC (Potters Bar, UK). Based on the current rate of use of TE-3, the batch of 13/240 is expected to last for a minimum of 25 years.

Collaborative Study

An international collaborative study was coordinated by NIBSC (under code CS515) and EDQM (under code BSP140). The study had 2 objectives:

1. Calibration of 13/240 in IU in terms of TE-3, using a toxin neutralisation test (TNT) in mice
2. Assessment of the performance of 13/240 in immunoassays that are used to measure the potency of TIg preparations

The study was launched in August 2016 and participants were asked to perform one or more of the following methods:

1. Toxin neutralisation test in mice
2. ELISA
3. Toxin binding inhibition assay (ToBI)

Based on the responses received, participants were provided with sufficient ampoules of TE-3 and 13/240, along with instructions for use, to enable them to perform at least 2 independent assays for method 1, and at least 3 independent assays for methods 2 and/or 3. A total of 20 laboratories (from China, Denmark, France, Germany, Hungary, India, Indonesia, Italy, Japan,

Republic of Korea, The Netherlands, Poland, Spain, UK and the USA) participated in the collaborative study. Participants were randomly assigned a code number. The participating laboratories are listed alphabetically by country, not according to the laboratory code number, at the end of this report. Details of the methods performed by study participants is summarised in Table 5.

Data Analysis

For all assay methods, all raw data together with assay details were provided to NIBSC to permit independent analysis: 21 valid data sets were returned by participants performing the mouse bioassay (8 laboratories); 56 valid data sets were returned by participants performing ELISA (14 laboratories); 11 valid data sets were returned by participants performing a ToBI assay (3 laboratories).

Method 1. Toxin neutralisation in mice
Data from all assays were analysed by probit parallel-line bioassay analysis comparing transformed assay responses to log dose using CombiStats 5.0 software [3]. For all assays, data for preparation 13/240 were analysed against TE-3 (120 IU/ampoule) and the resulting potency estimates are therefore based on direct pair-wise comparisons. Linearity and parallelism were assessed by analysis of variance, using the 1% level as a threshold for significance. In many cases, where the responses go between 0% and 100% in a single dilution step, the Spearman-Karber method was used to calculate potency estimates.

Method 2. ELISA
Relative potency estimates were calculated by fitting a parallel-line model [3] based on a linear section of the response range using a minimum of three dilutions and a log-transformation of the response. The only exception to this was laboratory 19a where the full range of data was used to fit a sigmoid (4-parameter logistic) model. Non-linearity and non-parallelism were considered in the assessment of assay validity. All data were plotted and a visual assessment was used to confirm linearity. Parallelism was confirmed by calculation of the ratio of fitted slopes for the test and reference samples under consideration and checking that this was within 0.80 to 1.25.

Method 3. Toxin binding inhibition (ToBI)
In nearly all laboratories the full range of data was used to fit a sigmoid (4-parameter logistic) model and calculate relative potencies. The exception to this was laboratory 17 where a parallel-line model [3], based on a linear section of the log-transformed response range using a minimum of three dilutions for all samples, was fitted. Parallelism was confirmed by calculation of the ratio of fitted slopes for the test and reference samples under consideration and checking that this was within 0.80 to 1.25.

Summary calculations
Unweighted geometric mean values were calculated for the laboratory means [3]. Overall means were calculated as unweighted geometric means of laboratory means. Variability within and between laboratories has been expressed using geometric coefficients of variation (GCV = $\{10^{s}-1\} \times 100\%$ where s is the standard deviation of the $\log_{10}$-transformed potency estimates). In order to mitigate the effect of any outliers or anomalous results, Huber's robust geometric mean was also calculated using the R package 'WRS2' [4].

Stability studies

The stability of the candidate standard 13/240 was determined by an accelerated degradation study using ampoules of 13/240 that had been held at elevated temperature for up to 4 years. Tetanus potency was determined by ELISA with potency estimates expressed relative to the response obtained for ampoules held at the recommended storage temperature of -20°C. At the 2.5-year time point, potency was also determined by mouse bioassay. All stability study assays were performed at NIBSC. The relative potencies of the accelerated thermal degradation samples were used to fit an Arrhenius equation relating degradation rate to absolute temperature assuming first-order decay [5], and hence predict the degradation rates when stored at a range of temperatures.

Results

Assay validity

Valid estimates of relative potency were obtained in almost all cases. Exceptions were two ELISAs by laboratory 14 and one ToBI assay by laboratory 12, where non-parallelism was observed, and one mouse TNT by laboratory 17 where the model could not be fitted due to a lack of convergence in the analysis.

Relative potency of 13/240 vs. TE-3

The results for individual assays performed by each laboratory are shown in Tables 6, 7 and 8 for the Mouse TNT, ELISA and ToBI assays, respectively. The within-laboratory variability was very low for all ELISA and ToBI assays with GCV ranging from 1.5% to 11.0%. Within-laboratory variability was not calculated for the mouse TNT because most participants performed only 2 independent assays as requested. The overall potency estimates obtained for 13/240 are comparable between the three different assay methods and the agreement between laboratories (for each method) is very good, as shown by between-laboratory GCV ranging from 6.4% to 12.9%. A summary of the overall relative potency estimates from each assay method is shown in Table 9 and Figure 1. The between laboratory variability is notably lower than for the previous collaborative study to calibrate TE-3 [1] and this is most likely due to the fact that the comparison in this study is human vs. human, whereas the comparison in the previous study was human vs. equine. Value assignment for TE-3 and previous WHO ISs for tetanus antitoxin has been based on the results obtained in the in vivo toxin neutralisation assay. The robust geometric mean for the mouse TNT assays is 44.9 IU/ampoule, which is comparable to the value from all assay methods included in the study (44.8 IU/ampoule).

Stability of 13/240

The potency of 13/240 was determined by ELISA at 7 time points over a 4-year period and the results obtained are shown in Table 10. All of this data was used to make a prediction of long-term stability by fitting an Arrhenius equation relating degradation rate to absolute temperature assuming first-order decay. The results of this analysis suggest that 13/240 will be extremely stable with no loss of activity predicted when the material is stored as recommended at -20°C in the dark. Even if stored at a slightly higher temperature of +2°C -+8°C, the results from the stability study suggest that 13/240 would not lose activity. The results from a single in vivo study (mouse bioassay) at the 29-month time point support these conclusions and there was no difference in the estimated potency for 13/240 held at +20°C for 29 months compared to the ampoule held at -20°C (Table 11).

Conclusion

The preparation of TIg coded 13/240 was successfully freeze-dried and meets all criteria for quality with respect to the precision of fill, residual moisture and long-term stability. The results of an international collaborative study suggest that 13/240 will be suitable for use in assays commonly used to measure the potency of tetanus immunoglobulin preparations and the potency estimates obtained in the study were comparable between laboratories and for all 3 assay methods included in the study. The results of a separate commutability study, described in an appendix to this report, provide good evidence that the candidate standard is commutable with human serum samples and therefore suitable for calibration of immunoassays that are used to determine anti-tetanus antibody titres in human serum. The candidate standard coded 13/240 is suitable for replacement of TE-3.

Recommendation

Product coded 13/240 is recommended as a replacement for TE-3 and for adoption as the 2nd WHO International Standard. It is recommended that the product coded 13/240 is assigned a value of **45 IU/ampoule**, for all assay methods, based on the potency determined in the mouse bioassay. The same recommendation will be made for establishment of 13/240 as the Ph. Eur. Biological Reference Preparation (BRP) for Human Tetanus Immunoglobulin, Batch 2.

Comments from study participants

The collaborative study report was sent to all study participants who were asked to acknowledge the conclusions and proposal and to comment on the content of the report where necessary. Eleven of 18 participants (not including the coordinating laboratories at NIBSC and EDQM) responded to acknowledge the report and all agreed with the conclusion and proposal.

Acknowledgements

We thank all the participants of the collaborative study as well as the donator of the starting material used in the production of the candidate standard. We thank Paul Matejtschuk and the staff of the Standards Processing Division, NIBSC, for the production of 13/240. The present study was coded CS515 for WHO/NIBSC and BSP140 for the EDQM/BSP. The study was run for EDQM under the aegis of the BSP of the Council of Europe and the EU Commission. Paul Stickings was nominated Project Leader of the BSP140 study by the Steering Committee of the BSP. The study was coordinated for the EDQM/BSP by Eriko Terao.

References

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Abbreviations

BRP: Biological Reference Preparation; BSP: Biological Standardisation Programme; IS: International Standard; IU: International Unit; Ph. Eur.: European Pharmacopoeia; TIg: Tetanus Immunoglobulin; TNT: Toxin Neutralisation Test; ToBI: Toxin Binding Inhibition assay; WHO: World Health Organization; CV: Coefficient of Variation; GCV: Geometric Coefficient of Variation; GM: Geometric Mean.

Table 1. Characteristics of source material and results of quality control tests

Parameter	Result
Batch No.	1049
Total volume	7.5 L
Date of manufacture	09 Sep 2013
Appearance	Complies
pH	6.6
Total protein content	154.5 g/L
Protein composition	97.6%
Molecular size distribution	<3% polymer/aggregate
Glycine content	21.2 g/L
Sterility	Sterile
Pyrogens	Pyrogen free
Tetanus potency	148.7 IU/mL

Table 2. Summary of freeze-dried product 13/240 – fill and freeze drying specifications

Parameter	Specification	Result
Precision of fill	<0.25% CV	0.16% CV (n=603)
Mean residual moisture	<1%	0.38% (n=12)
Mean oxygen headspace	<1.14%	0.41% (n=12)

Table 3. Summary of freeze-dried product 13/240 – post fill characterisation

Parameter	Result
Appearance	Good robust cake
Reconstitution	Rapid and complete
Tetanus potency (ELISA)	Liquid bulk = 53.8 (49.2 – 58.9) IU/mL 13/240 = 50.4 (49.2 – 51.6) IU/mL
Tetanus potency (mouse bioassay)	Liquid bulk assay 1 = 49.2 IU/mL Liquid bulk assay 2 = 55.0 IU/mL 13/240 assay 1 = 46.0 IU/mL 13/240 assay 2 = 45.5 IU/mL
Molecular size distribution (SE-HPLC)	<3% aggregates

Table 4. Tetanus potency for ampoules of 13/240 taken from across the filling run

Ampoule No.	**ELISA potency IU/mL relative to TE-3**			
	Plate 1	Plate 2	Plate 3	GM (95% Confidence Interval)
1	52.6	54.2	48.9	51.9 (45.5 – 59.1)
2	54.1	53.8	46.5	51.3 (41.5 – 63.6)
3	55.8	46.3	52.9	51.5 (40.6 – 65.4)
4	53.6	48.7	50.4	50.9 (45.1 – 57.4)
5	50.9	47.4	52.0	50.1 (44.4 – 56.5)
6	46.1	50.4	50.9	49.1 (42.9 – 56.2)
7	45.7	49.1	51.1	48.6 (42.2 – 55.9)
8	49.6	46.5	47.9	48.0 (44.3 – 52.0)
9	50.5	53.2	53.5	52.4 (48.4 – 56.7)
Liquid bulk	51.7	55.4	54.5	53.8 (49.2 – 58.9)

Table 5. Summary of methods performed by participating laboratories

Lab Code	Assay Method	Method identifier	Tetanus Antigen (ELISA/ToBI)	Toxin dose level (mouse TNT)	No. assays performed
1	ELISA	In-house	Tetanus toxoid (NIBSC 04/150)	N/A	3
2	ELISA	Commercial kit	Tetanus toxoid	N/A	3
3	Mouse TNT	In-house	N/A	Not stated	5
4	Mouse TNT	Ph. Eur.	N/A	Lp/10	2
5	ELISA	In-house	Tetanus toxoid (NIBSC 02/126)	N/A	3
6	ELISA	Commercial kit	Tetanus toxoid	N/A	3
7	ELISA	Commercial kit	Tetanus toxoid	N/A	3
8	ELISA	Commercial kit	Not stated	N/A	3
8	ELISA	Commercial kit	Not stated	N/A	3
9	Mouse TNT	ChP 2015	N/A	L+/10	4
10	ToBI	In-house	WHO tetanus toxoid	N/A	3
11	ELISA	Commercial kit	Tetanus toxoid	N/A	3
12	ELISA	In-house	Tetanus toxoid (NIBSC 02/126)	N/A	6
12	ToBI	In-house	Tetanus toxoid (NIBSC 02/126)	N/A	6
13	Mouse TNT	In-house	N/A	L+/10	2
14	ELISA	Commercial kit	Tetanus toxoid	N/A	3
14	Mouse TNT	In-house	N/A	L+/10	2
15	Mouse TNT	In-house	N/A	L+/10	2
16	ELISA	In-house	Tetanus toxoid (NIBSC 02/232)	N/A	3
17	ELISA	Commercial Kit	Tetanus toxoid	N/A	3
17	Mouse TNT	B.P.	N/A	L+/10	3
18	ELISA	In-house (Ph. Eur. 0398)	Tetanus toxoid (NIBSC 04/150)	N/A	3

18	ToBI	In-house	Tetanus toxoid (NIBSC 04/150)	N/A	3
19	ELISA	In-house (Ph. Eur. 0398)	Tetanus toxoid (NIBSC 02/126)	N/A	13
19	Mouse TNT	In-house	N/A	Lp/200	3
20	ELISA	Commercial kit	Not stated	N/A	3

Table 6. Collaborative study results (Mouse TNT)

Lab Code	Assay Number					Laboratory Geometric Mean
	1	2	3	4	5	
3	40.0	38.9	38.6	41.3	45.4	40.8
4	50.0	41.5				45.6
9	44.7	45.9	44.7	45.4		45.2
13	43.4	44.4				43.9
14	45.8	50.0				47.9
15	39.0	40.0				39.5
17	47.7	NE	45.0			46.3
19	46.0	45.5	50.0			47.1

Data is the potency (IU/ampoule) for 13/240 relative to TE-3; NE = No estimate as model could not be fitted

Table 7. Collaborative study results (ELISA)

Lab Code	Assay Number										Lab Geometric Mean	Lab GCV %
	1	2	3	4	5	6	7	8	9	10		
01	51.1	41.8	46.9								46.4	10.6
02	53.7	51.8	63.0								56.0	11.0
05	36.7	40.0	37.4								38.0	4.6
06	41.6	42.3	47.0								43.6	6.8
07	38.3	41.6	44.0								41.2	7.2
08a	38.6	43.5	38.4								40.1	7.3
08b	43.5	37.9	44.8								42.0	9.3
11	41.5	44.1	42.0								42.5	3.3
12	46.8	48.2	46.5	47.9	49.6	50.4					48.2	3.2
14	45.5*	53.9	54.4*								53.9	N/A
16	34.8	39.2	40.3								38.0	8.1
17	40.2	39.8	41.0								40.3	1.5
18	49.7	49.3	51.5								50.1	2.3
19a	48.8	48.8	51.2								48.3	8.3
19b	51.1	41.9	49.1	45.0	47.7	40.7	51.0	50.6	50.7	52.8		
20	42.6	43.4	37.4								41.0	8.4

Data is the potency (IU/ampoule) for 13/240 relative to TE-3; * = non-parallel (not included in laboratory final estimate); lab code 08a and 08b are the same participant performing two different commercial ELISA methods (not combined for the laboratory estimate); lab code 19a

and 19b are the same participant performing the same ELISA method but in two separate periods (laboratory estimate is the combination of both)

Table 8. Collaborative study results (ToBI)

Lab Code	Assay Number						Laboratory Geometric Mean	Lab GCV%
	1	2	3	4	5	6		
10	43.7	45.0	50.1				46.2	7.5
12	51.1	51.3	52.2	54*	53.2	53.7	52.3	2.2
18	48.3	50.6	49.1				49.3	2.4

Data is the potency (IU/ampoule) for 13/240 relative to TE-3; * = non-parallel (not included in laboratory final estimate)

Table 9. Overall summary of potency estimates for 13/240 (IU/ampoule) relative to TE-3

	Assay Method			
	Mouse TNT	**ELISA**	**ToBI**	**All methods**
Geometric Mean	44.4	44.3	49.2	44.9
GCV	6.8%	12.9%	6.4%	11.0%
n	8	15	3	26
95% C.I.	(42.0 – 47.1)	(41.5 – 47.4)	(42.2 – 57.4)	(43.0 – 46.8)
Robust Geometric Mean	**44.9**	**44.0**	**49.2**	**44.8**

Table 10. Stability of 13/240 (accelerated degradation study using ELISA)

Time point (month)	Potency relative to ampoule held at -20°C for each elevated temperature			
	+4°C	+20°C	+37°C	+45°C
1	1.05 (1.03 – 1.06)	1.05 (1.03 – 1.06)	1.15 (1.14 – 1.17)	1.04 (1.02 – 1.05)
3	0.96 (0.93 – 1.00)	0.95 (0.92 – 0.98)	1.07 (0.97 – 1.17)	1.17 (1.08 – 1.27)
6	1.01 (0.98 – 1.04)	0.94 (0.91 – 0.97)	0.96 (0.93 – 0.99)	ND
12	0.97 (0.92 – 1.02)	1.04 (0.99 – 1.10)	1.19 (1.09 – 1.30)	0.95 (0.85 – 1.06)
29	0.96 (0.94 – 0.98)	0.98 (0.94 – 1.01)	1.10 (1.05 – 1.15)	ND
39	1.17 (1.14 – 1.21)	1.12 (1.08 – 1.15)	ND	ND
50	0.94 (0.91 – 0.96)	0.91 (0.87 – 0.95)	ND	ND

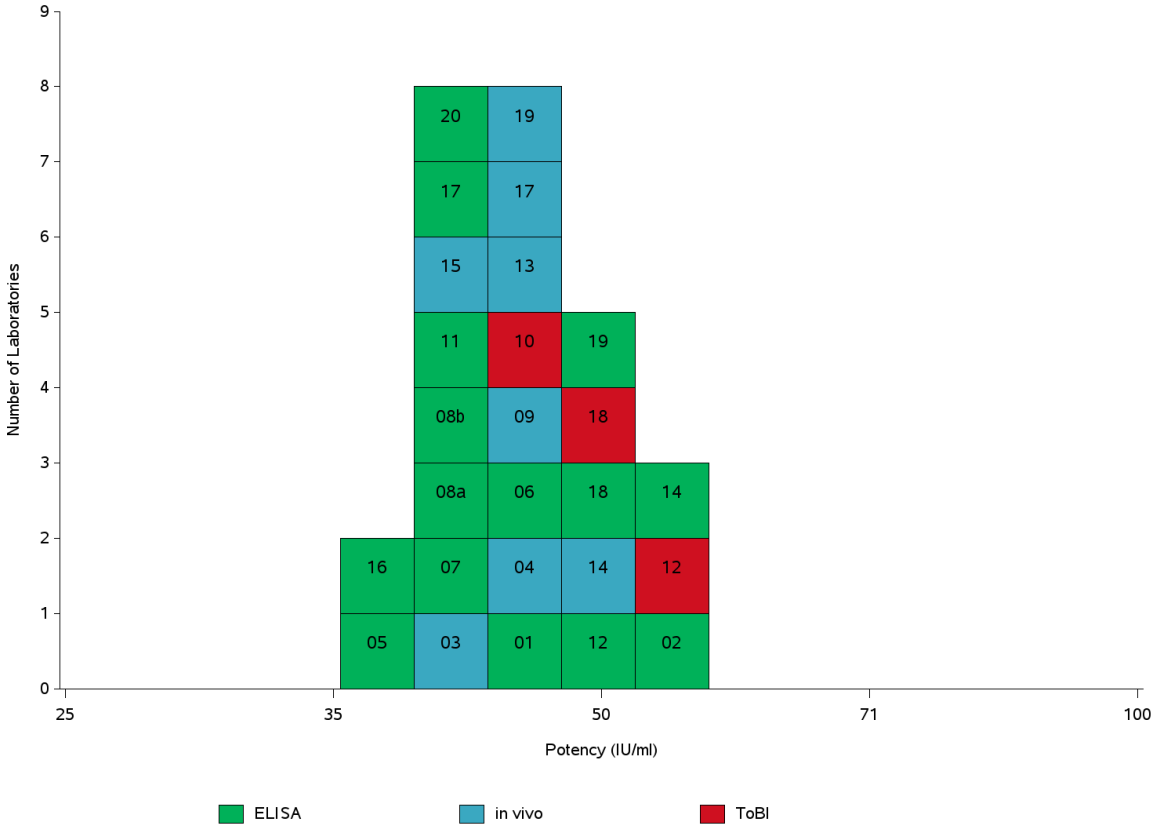
Data is potency for ampoules of 13/240 held at elevated temperature, relative to ampoules held under recommended storage conditions of -20°C in the dark; the result for each time point is the combined estimate from n=3 ELISA plates with 95% Confidence Interval; ND = not done because of incomplete reconstitution of freeze-dried material following extended storage at high temperature

Table 11. Stability of 13/240 (accelerated degradation study using Mouse TNT)

Time point (month)	Potency relative to ampoule held at -20°C for each elevated temperature			
	+4°C	+20°C	+37°C	+45°C
29	88.4%	100%	78.6%	Not included

Data is potency (expressed as a %) for ampoules of 13/240 held at elevated temperature, relative to ampoules held under recommended storage conditions of -20°C in the dark

Figure 1. Histogram showing relative potency estimates for 13/240 vs. TE-3



Data is the laboratory geometric mean relative potency estimate for 13/240 vs. TE-3 (IU/ampoule)

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Appendix A
Commutability study for the candidate 2nd International Standard for Tetanus Immunoglobulin Human

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Introduction

The WHO International Standard for Tetanus Immunoglobulin Human is used for calibration of assays that measure the concentration of anti-tetanus antibodies in human serum. This includes a number of commercial diagnostic immunoassay kits. Therefore, as part of the study to characterise the proposed 2nd WHO IS for Tetanus Immunoglobulin Human (coded 13/240), NIBSC conducted an assessment of commutability using commercial and in-house ELISA methods and a large panel of human serum samples.

Commutability is a property of a reference material that can be defined as: "the equivalence of the mathematical relationships among the results of different measurement procedures for a reference material and for representative samples of the type intended to be measured" [1]. Commutability is a critical property of a reference preparation to ensure that it is suitable for its intended use and is particularly important for reference preparations that are intended to calibrate diagnostic assays.

The commutability study was conducted over two phases. In phase I, serum samples were tested in 10 different assay methods (8 commercial tetanus ELISA kits and 2 in-house tetanus ELISAs). Some evidence of a possible dilution effect was observed during phase I so a follow up study was conducted (phase II) using a smaller panel of samples and a smaller number of methods, and including additional dilutions of the reference preparations and additional dilutions of selected high titre serum samples.

Materials and Methods

Samples used in the study

Assessment of commutability was performed using a panel of individual "patient" samples that covered a range of anti-tetanus antibody titres. The human serum samples (n=38) were obtained from Cerba Specimen Services (Saint Ouen L'Aumône, France) supplemented with 6 human serum samples from NIBSC. A summary of these 44 human serum samples is shown in Table A1. Serum samples obtained from Cerba were received as frozen aliquots. Prior to use, these samples were thawed and divided into a panel of smaller aliquots and re-frozen at -20°C. For each assay performed, one of these aliquots was removed for each sample and thawed at room temperature prior to dilution and use in the ELISA. All serum samples were diluted 1/100 for testing by ELISA.

Some high titre samples were further diluted to ensure that the assay response fell within the range of the standard curve used in the ELISA assays.

In addition to the candidate 2nd WHO IS for Tetanus Immunoglobulin Human (coded 13/240) and its liquid comparator prior to freeze-drying (coded Bulk TIg), some other reference preparations were also included in the study: the current (1st) WHO IS for Tetanus Immunoglobulin Human (coded TE-3); the current (1st) WHO IS for Diphtheria Antitoxin Human (coded 10/262); a working standard tetanus anti-serum from NIBSC (coded 76/589).

ELISA methods used in the study
For phase 1, all samples (and candidate/established reference preparations) were tested using eight commercial ELISA kits (coded A-H) and two in-house ELISA assays (coded I-J). For phase 2, a subset of 20 serum samples and the candidate 2nd WHO IS for Tetanus Immunoglobulin Human (coded 13/240) were tested using five of the commercial ELISA kits and the two in-house ELISAs. Commercial ELISAs were performed according to manufacturer's instructions. For the NIBSC in-house ELISAs, 96-well maxisorb ELISA plates were coated with 0.5 Lf/ml tetanus toxoid (NIBSC code 02/126, method code I) or tetanus toxin (in-house tetanus toxin AWX4664, 1/20 dilution, method code J) in carbonate coating buffer (100 µl per well, overnight at +2-8°C). After washing and blocking plates with 5% skimmed milk powder in PBS-Tween (0.05%), serum samples (diluted in 1% skimmed milk powder in PBS-Tween (0.05%) were added to the plate (100 µl per well). The 1st WHO IS for Tetanus Immunoglobulin Human (TE-3) was titrated in duplicate as the reference antitoxin (3-fold titration starting at 0.05 IU/ml, 100 µl per well). Plates were incubated at +37°C for 2h prior to washing and addition of 100 µl per well of rabbit anti-human IgG HRP conjugate (Sigma, A8792, 1/2000 dilution). Following a 1h incubation at +37°C, ABTS substrate solution was added and the absorbance read at 405 nm.

Data used for analysis. All results were $\log_{10}$ transformed for analysis in order to achieve approximately constant variance over the range of sample concentrations tested. Consensus values for each sample, shown in Tables A2 and A5, were calculated as Huber's robust mean of laboratory means using the R package 'WRS2' [2]. Bias values were then calculated for all individual results as the difference between the result and the study consensus value for that sample.

Determination of commutability criteria. The robust mean and standard deviation of the bias values for the serum samples (undiluted only) were calculated for each assay method using R package 'WRS2' and the median standard deviation value, s_M, was used to set commutability criteria as $\pm 2s_M$, representing the maximum acceptable difference in bias, i.e. other preparations were to be concluded as commutable for a particular assay method if their observed bias was within $\pm 2s_M$ of that observed for serum samples for that method.

Assessment of inter-method variability. In order to quantify inter-method variability in geometric mean results for each sample, geometric coefficients of variation (defined as GCV = $\{10^{s}-1\}\times100\%$ where s is the standard deviation of the $\log_{10}$ transformed estimates) were calculated using reported results directly and using results expressed relative to TE-3 or 13/240 for each method.

Results – Phase I

In order to achieve approximately constant bias and ensure that the serum samples and diluted standards gave results in the same range of concentrations, samples with consensus values outside the range 0.15 to 3.31 IU/ml were excluded from further analysis (these are indicated with * in Table A2).

Commutability criteria calculated using $2s_M$ were ±0.152, or 0.70 to 1.42 on the untransformed scale, i.e. the bias for a reference preparation must be demonstrated to be not less than 70% and not more than 142% of the bias observed for serum samples.

Estimates of the difference in bias are shown in Table A3 and comparisons of these values with the commutability criteria are shown in Figure A1. Mean bias estimates for individual samples and dilutions are shown in Figure A2. With the exception of working standard coded 76/589 tested by method A, which gave values outside the commutability criteria at all dilutions tested, the majority of dilutions of all reference preparations were within the defined commutability criteria (95% of cases). In the small number of cases (5%) with a result outside the criteria, this was generally noted to be at the lowest or highest dilution of the sample tested. Due to this observation, and the suggestion of non-constant bias with dilution for some of the samples tested, a follow up Phase II study was performed.

A comparison of inter-method variability for the serum sample results as reported or expressed relative to TE-3 (current WHO IS) or 13/240 (candidate replacement WHO IS) is shown in Table A4. No GCV differences greater than ±1% were observed for any of the serum samples tested and a pooled GCV of ~29% is obtained for each of the columns in Table A3, demonstrating no negative impact to the harmonisation of serum sample results when expressed relative to the candidate replacement WHO IS 13/240.

Results – Phase II

The results obtained in phase I indicated that there was non-constant bias with dilution for some of the samples tested. As a result, a follow up study was conducted using a smaller selection of ELISA methods (commercial ELISA kits B, C, D, F, H and in-house ELISAs I-J) and a smaller panel of serum samples (n=20). In this follow up study the candidate 2nd WHO IS for Tetanus Immunoglobulin Human (coded 13/240) was the only reference preparation included for

commutability assessment. As part of the serum panel, a small number of high titre serum samples were additionally included with further dilutions to identify whether any observed (dilution) effect for the candidate reference preparation was also observed for patient samples.

The consensus values obtained for each sample is shown in Table A5. The assumption of constant bias appeared reasonable for all methods, but not for serum samples with low concentrations, so samples with consensus values ≤ 0.10 IU/ml were excluded from further analysis. Any diluted samples with consensus values outside the range observed for the individual serum samples (0.11 to 2.66 IU/ml) were also excluded from further analysis (excluded samples are indicated with * in Table A5).

Commutability criteria of ±0.152 determined using data from Phase I were also applied to the results from Phase II. Recalculation of the criteria gave a slightly wider range of ±0.177 using the data from Phase II only.

Estimates of the difference in bias are shown in Table A6 and comparisons of these values with the commutability criteria are shown in Figure A3. Mean bias estimates for individual samples and dilutions are shown in Figure A4. The majority of dilutions of all samples were within the defined commutability criteria (96% of cases). The small number of cases (4%) with a result outside the criteria corresponded to dilutions of serum samples 29 and 30, with no observed cases for the candidate replacement International Standard 13/240.

Fitted slopes calculated by linear regression of bias values on log consensus value are shown in Table A7, with those showing an absolute value greater than 0.10 highlighted. Where an absolute slope exceeding 0.10 was observed for 13/240 for methods H and I, this was also observed for two of the three serum samples tested at multiple dilutions, suggesting any lack of constant bias with dilution is not a unique property of the reference preparation 13/240. Fitted slopes for 13/240 showed similar outcomes for the diluted serum samples for all of the methods performed.

A comparison of inter-method variability for serum sample results as reported or expressed relative to 13/240 is shown in Table A8. No GCV differences greater than ±2% were observed for any of the serum samples tested and a pooled GCV of ~31% is obtained for each of the columns in Table A8, demonstrating no negative impact to the harmonisation of serum sample results following the introduction of replacement IS 13/240. This is consistent with the result obtained in Phase I of the commutability study.

Conclusion

The candidate 2nd WHO IS for Tetanus Immunoglobulin Human (coded 13/240) shows comparable behaviour to human serum samples across a range of tetanus ELISA assays. Where evidence of a dilution effect was observed, the same was also seen for diluted high titre serum

samples suggesting that this effect is not unique to the candidate standard and that the candidate standard behaves in a similar way to these serum samples. The results obtained in this commutability study provide good evidence that 13/240 is commutable with human serum samples and that it will be suitable for calibration of immunoassays that are intended for measurement of anti-tetanus antibody titres. Results obtained from a large panel of human serum samples confirm that the inter-method variability is comparable when results are expressed relative to 13/240 or to the current standard (TE-3) or to the internal kit standard used in commercial ELISA kits. This provides additional evidence of the suitability of 13/240 and suggests that there will be no negative impact to the harmonisation of serum sample results when expressed relative to this reference preparation.

In addition to an assessment of the commutability of the candidate replacement standard 13/240, this study also provided an opportunity to assess commutability for some other reference preparations (in Phase I). This included the current WHO IS for Tetanus Immunoglobulin Human (TE-3) for which commutability was not assessed when it was first established in 1992. Results from this study show that this standard is also commutable with human serum samples with respect to anti-tetanus antibody titres.

Table A1. Summary of human serum samples used in the commutability study together with the reported anti-tetanus titre that was obtained prior to the study. NR = not reported.

Sample No.	Source	Gender	Age	Reported anti-tetanus titre IU/ml	Storage conditions
1	Cerba	Female	5	0.95	Frozen – 20°C
2	Cerba	Male	11	1.03	Frozen – 20°C
3	Cerba	Female	15	1.03	Frozen – 20°C
4	Cerba	Male	2	0.05	Frozen – 20°C
5	Cerba	Male	41	2.46	Frozen – 20°C
6	Cerba	Female	1	>5.00	Frozen – 20°C
7	Cerba	Male	64	0.09	Frozen – 20°C
8	Cerba	Female	57	1.37	Frozen – 20°C
9	Cerba	Male	48	2.08	Frozen – 20°C
10	Cerba	Male	4	0.16	Frozen – 20°C
11	Cerba	Female	17	0.06	Frozen – 20°C
12	Cerba	Female	16	0.13	Frozen – 20°C
13	Cerba	Female	43	>5.00	Frozen – 20°C
14	Cerba	Female	46	0.06	Frozen – 20°C
15	Cerba	Male	72	0.25	Frozen – 20°C

16	Cerba	Male	30	0.36	Frozen – 20°C
17	Cerba	Female	44	0.10	Frozen – 20°C
18	Cerba	Male	48	0.10	Frozen – 20°C
19	Cerba	Female	38	0.53	Frozen – 20°C
20	Cerba	Female	79	>5.00	Frozen – 20°C
21	Cerba	Female	91	0.09	Frozen – 20°C
22	Cerba	Male	50	0.07	Frozen – 20°C
23	Cerba	Female	77	0.16	Frozen – 20°C
24	Cerba	Female	52	1.68	Frozen – 20°C
25	Cerba	Male	62	0.87	Frozen – 20°C
26	Cerba	Male	45	1.07	Frozen – 20°C
27	Cerba	Female	53	0.04	Frozen – 20°C
28	Cerba	Female	30	0.16	Frozen – 20°C
29	Cerba	Female	24	>5.00	Frozen – 20°C
30	Cerba	Male	31	>5.00	Frozen – 20°C
31	Cerba	Male	48	>5.00	Frozen – 20°C
32	Cerba	Male	56	3.75	Frozen – 20°C
33	Cerba	Male	18	4.89	Frozen – 20°C
34	Cerba	Female	71	3.06	Frozen – 20°C
35	Cerba	Male	12	>5.00	Frozen – 20°C
36	Cerba	Male	2	3.06	Frozen – 20°C
37	Cerba	Male	1	>5.00	Frozen – 20°C
38	Cerba	Male	29	3.31	Frozen – 20°C
39	NIBSC	NR	NR	0.08	+2-8°C
40	NIBSC	NR	NR	0.85	+2-8°C
41	NIBSC	NR	NR	1.07	+2-8°C
42	NIBSC	NR	NR	2.49	+2-8°C
43	NIBSC	NR	NR	10.70	+2-8°C
44	NIBSC	NR	NR	1.88	+2-8°C

Table A2. Phase I. Consensus values obtained for all samples. Values in brackets for the reference preparations indicate dilution; *indicates samples that were excluded from further analysis.

Sample	Robust Mean $\log_{10}$ IU/ml	Robust Geometric Mean IU/ml	Sample	Robust Mean $\log_{10}$ IU/ml	Robust Geometric Mean IU/ml
Serum 1	-0.03	0.93	Serum 32	0.36	2.30

Serum 2	-0.11	0.78	Serum 33	0.27	1.84
Serum 3	-0.06	0.87	Serum 34	0.21	1.63
Serum 4*	-1.20	0.06	Serum 35	0.25	1.78
Serum 5	0.29	1.96	Serum 36	0.19	1.55
Serum 6	0.36	2.28	Serum 37	0.49	3.08
Serum 7*	-0.96	0.11	Serum 38	0.52	3.31
Serum 8	0.15	1.42	Serum 39*	-1.02	0.10
Serum 9	0.15	1.43	Serum 40	-0.41	0.39
Serum 10	-0.83	0.15	Serum 41	-0.14	0.72
Serum 11*	-1.09	0.08	Serum 42	0.26	1.83
Serum 12*	-0.99	0.10	Serum 43	0.19	1.55
Serum 13	0.22	1.65	Serum 44	0.16	1.43
Serum 14*	-1.04	0.09	Bulk TIG (10)	0.49	3.10
Serum 15	-0.68	0.21	Bulk TIG (50)	-0.07	0.85
Serum 16	-0.47	0.34	Bulk TIG (100)	-0.33	0.47
Serum 17	-0.81	0.15	13/240 (10)	0.49	3.09
Serum 18*	-0.91	0.12	13/240 (50)	-0.09	0.82
Serum 19	-0.37	0.42	13/240 (100)	-0.35	0.45
Serum 20	0.37	2.33	13/240 (250)	-0.72	0.19
Serum 21*	-0.89	0.13	TE-3 (20)*	0.62	4.18
Serum 22*	-0.99	0.10	TE-3 (100)	0.06	1.16
Serum 23	-0.82	0.15	TE-3 (200)	-0.21	0.62
Serum 24	0.28	1.89	TE-3 (500)	-0.56	0.27
Serum 25	-0.06	0.86	10/262 (10)	0.08	1.21
Serum 26	-0.14	0.73	10/262 (50)	-0.54	0.29
Serum 27*	-0.99	0.10	10/262 (100)	-0.79	0.16
Serum 28	-0.75	0.18	76/589 (2)	0.51	3.24
Serum 29	0.31	2.06	76/589 (10)	-0.10	0.80
Serum 30	0.44	2.78	76/589 (50)	-0.70	0.20
Serum 31	0.30	1.97	76/589 (250)*	-1.16	0.07

Table A3. Phase I. Estimated difference in bias with serum samples. Shading indicates values outside commutability criteria range.

Method Code	Dilution	Bulk TIg	13/240	TE-3	10/262	76/589
A	2					-0.250
	10	-0.041	-0.048		-0.018	-0.241
	50	-0.036	-0.048		-0.029	-0.365
	100	-0.015	-0.060	-0.043	0.014	
	200			-0.091		
	250		-0.041			
	500			-0.031		
B	10	-0.014	0.025		0.052	
	50	0.061	0.048		0.010	
	100	0.013	0.048	0.119	-0.022	
	200			0.050		
C	10	0.001	-0.011		0.045	
	50	0.036	0.028		0.000	
	100	0.020	0.037	0.062	-0.043	
	200			0.037		
D	10	0.055	0.005		-0.048	
	50	-0.028	-0.060		-0.087	
	100	-0.060	-0.031	-0.014	-0.139	
	200			0.162		
E	2					0.065
	10	-0.141	-0.117		-0.004	0.154
	50	0.026	0.004		-0.015	0.068
	100	0.038	0.002	0.003	-0.066	
	200			-0.004		
	250		-0.056			
	500			-0.001		
F	10	-0.077	-0.040		-0.033	
	50	-0.036	-0.016		-0.011	
	100	-0.009	-0.039	-0.059	0.014	
	200			-0.076		
G	2					-0.023
	10	0.010	0.038		-0.055	-0.127
	50	-0.013	-0.029		-0.129	-0.169
	100	-0.097	-0.116	-0.005	-0.083	
	200			-0.042		
	250		-0.188			
	500			-0.156		

H	2					-0.015
	10	-0.044	-0.035		0.037	0.037
	50	0.007	0.041		0.110	0.084
	100	0.048	0.061	0.054	0.156	
	200			0.027		
	250		0.065			
	500			0.045		
I	10	0.180			0.038	0.043
	50	0.018	0.049		0.141	0.121
	100	0.062	0.078	0.049	0.166	
	200			0.072		
	250		0.097			
	500			0.102		
J	2					0.057
	10	0.023	-0.034		0.015	0.089
	50	0.111	0.040		0.025	0.061
	100	0.015	0.044	-0.083	0.017	
	200			-0.057		
	250		0.000			
	500			-0.109		

Figure A1. Phase I. Estimated difference in bias with serum samples for the different reference preparations included in the study. Dashed red lines indicate the range of acceptable difference in bias for commutable samples.

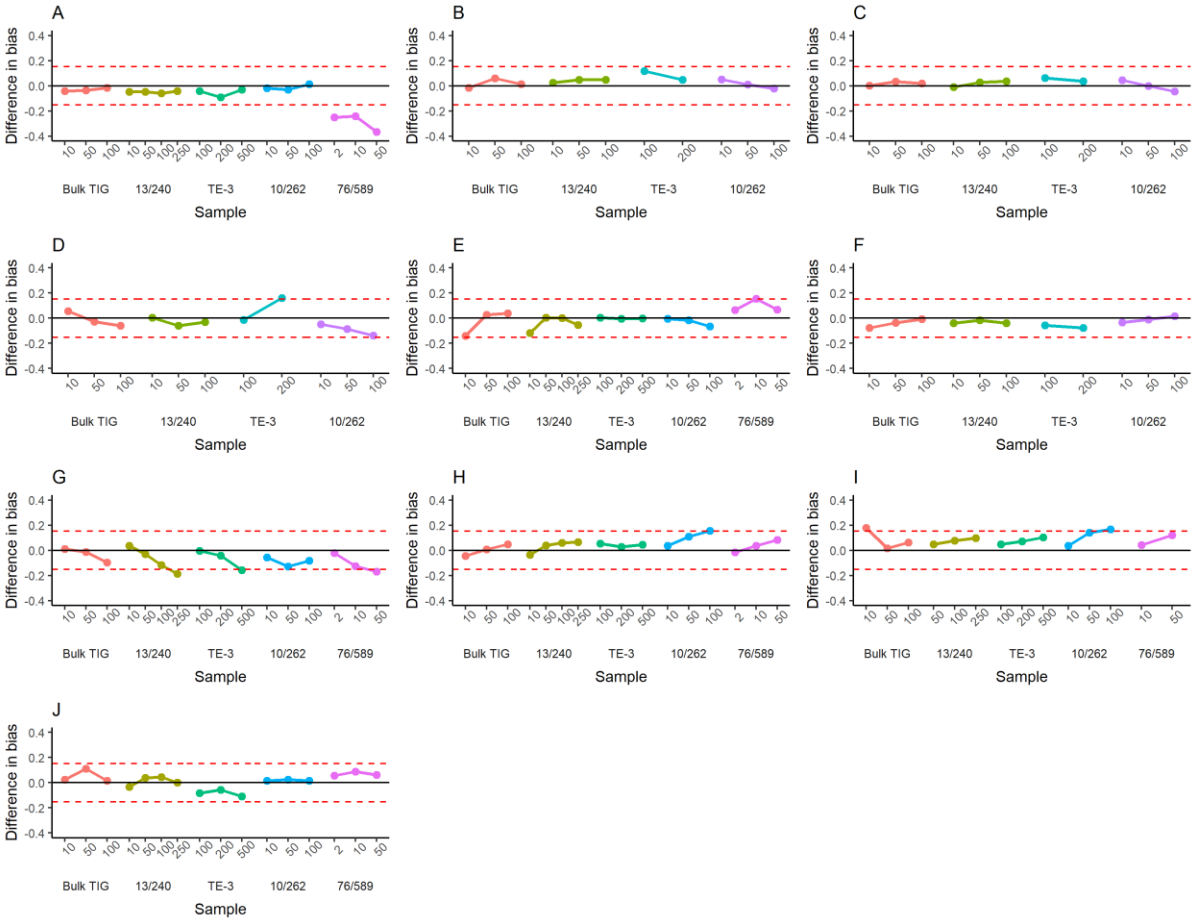

Figure A2. **Phase I**. Mean bias estimates for individual samples and dilutions. Dashed lines indicate mean method bias for serum samples (red line) and range of acceptable values for other preparations demonstrating commutability (black lines).

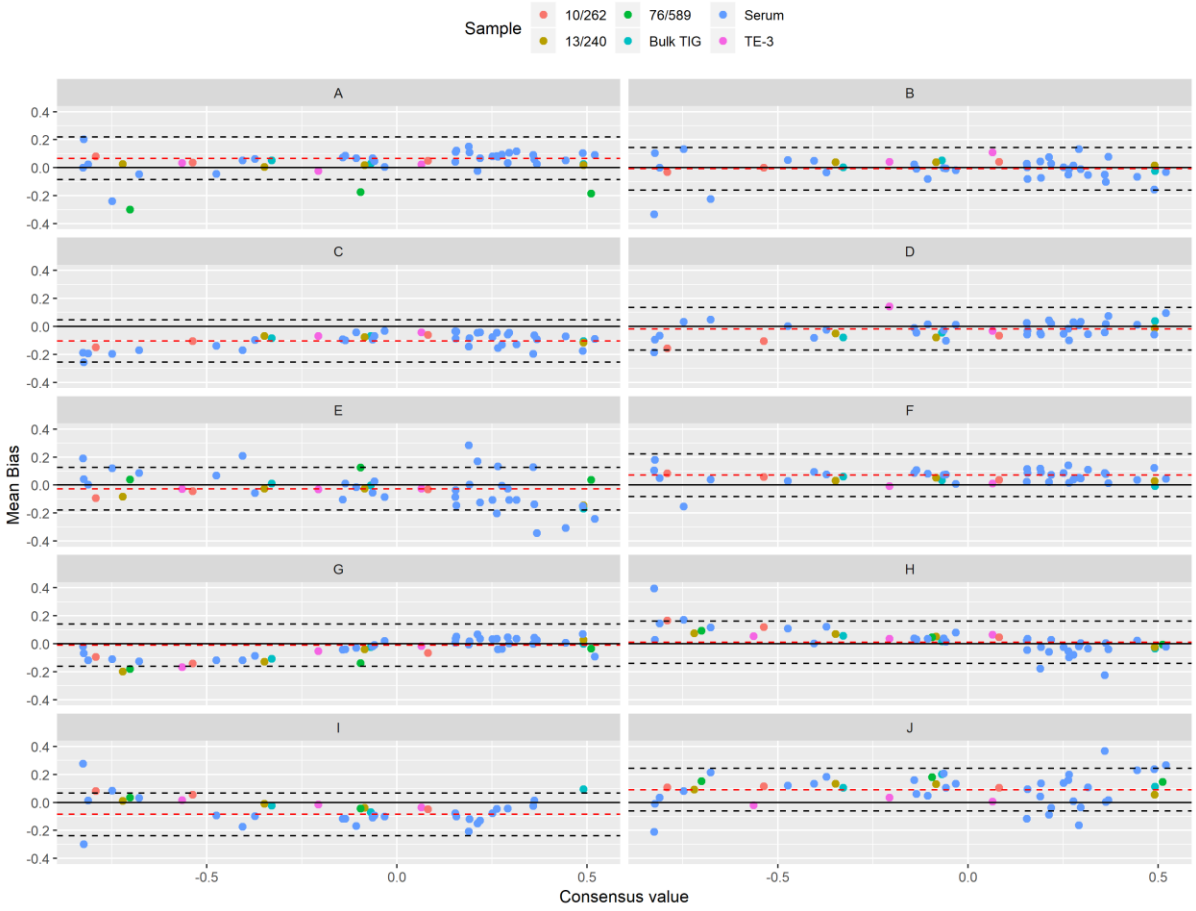

Table A4. Phase I. Inter-method variability in results for serum samples

Serum sample	Inter-method %GCV		
	Reported	Rel. to 13/240	Rel. to TE-3
1	17.5	17.7	17.7
2	19.1	19.7	19.4
3	17.4	17.6	17.2
5	20.0	20.2	20.7
6	47.7	47.5	46.9
8	14.1	14.5	15.0
9	19.3	18.9	19.5
10	72.6	72.0	72.1
13	18.4	18.8	19.3
15	37.1	36.8	36.3
16	24.0	24.2	23.7
17	24.3	23.9	24.0
19	25.9	25.9	25.8
20	34.6	34.2	35.2
23	47.0	47.5	47.3
24	16.8	16.5	16.6
25	23.9	23.9	23.8
26	18.8	19.1	18.9
28	41.7	41.4	41.1
29	24.6	24.1	24.6
30	38.6	38.0	38.6
31	15.0	14.7	15.6
32	17.8	17.3	18.0
33	30.7	30.4	29.8
34	23.7	24.6	24.0
35	20.7	20.6	20.8
36	43.1	43.8	43.0
37	42.8	42.2	42.5
38	39.4	38.9	39.3
40	35.3	35.8	34.9
41	23.3	23.4	23.5
42	28.1	27.8	28.3
43	22.5	22.7	22.4
44	23.5	23.6	23.9

Table A5. Phase II. Consensus values obtained for all samples. Values in brackets after samples indicate dilution; *excluded from further analysis.

Sample	Robust Mean log_{10} IU/ml	Robust Geometric Mean IU/ml	Sample	Robust Mean log_{10} IU/ml	Robust Geometric Mean IU/ml
Serum 1	-0.15	0.71	13/240 (5)*	0.64	4.38
Serum 2	-0.17	0.67	13/240 (12.5)	0.37	2.34
Serum 3	-0.16	0.69	13/240 (31.3)	0.04	1.09
Serum 5	0.22	1.65	13/240 (78.1)	-0.31	0.49
Serum 7*	-1.06	0.09	13/240 (195.3)	-0.67	0.21
Serum 12*	-1.06	0.09	13/240 (488.3)*	-1.00	0.10
Serum 15	-0.77	0.17	13/240 (772.1)*	-1.12	0.08
Serum 16	-0.54	0.29	13/240 (1220.7)*	-1.24	0.06
Serum 17	-0.86	0.14	29 (5)	-0.05	0.89
Serum 18	-0.97	0.11	29 (12.5)	-0.42	0.38
Serum 19	-0.44	0.36	29 (31.3)	-0.82	0.15
Serum 21	-0.90	0.13	29 (78.1)*	-1.05	0.09
Serum 24	0.21	1.62	30 (12.5)	0.06	1.14
Serum 29	0.25	1.78	30 (31.3)	-0.28	0.53
Serum 30	0.43	2.66	30 (78.1)	-0.62	0.24
Serum 34	0.18	1.53	30 (195.3)	-0.95	0.11
Serum 39*	-1.14	0.07	43 (6.3)	0.07	1.18
Serum 40	-0.47	0.34	43 (15.6)	-0.31	0.48
Serum 43	0.42	2.60	43 (39.1)	-0.64	0.23
Serum 44	0.04	1.10	43 (97.7)	-0.96	0.11

Table A6. Phase II. Estimated difference in bias with serum samples. Shading indicates values outside commutability criteria range.

Method Code	Dilution	13/240	Serum 29	Serum 30	Serum 43
B	5		0.008		
	6.3				-0.044
	12.5	-0.002	0.010	-0.041	
	15.6				-0.025
	31.3	-0.024	-0.009	-0.018	
	39.1				-0.036
	78.1	-0.016		-0.046	
	97.7				-0.109
	195.3	-0.001		-0.076	
C	5		-0.006		
	6.3				0.020
	12.5	0.057	-0.026	0.080	
	15.6				-0.027
	31.3	0.022	-0.128	0.042	
	39.1				-0.027
	78.1	0.013		0.013	
	97.7				-0.080
	195.3	-0.025		-0.052	
D	5		-0.086		
	6.3				-0.041
	12.5	-0.014	-0.176	-0.125	
	15.6				-0.072
	31.3	0.007	-0.235	-0.133	
	39.1				-0.088
	78.1	-0.015		-0.117	
	97.7				-0.090
F	5		0.083		
	6.3				0.094
	12.5	0.013	0.095	0.072	
	15.6				0.071
	31.3	-0.005	0.066	0.021	
	39.1				0.082
	78.1	-0.064		-0.010	
	97.7				0.120
	195.3	-0.053		0.026	
H	5		-0.016		

	6.3				-0.045
	12.5	-0.053	0.023	-0.003	
	15.6				-0.020
	31.3	-0.017	0.038	0.039	
	39.1				0.041
	78.1	0.022		0.105	
	97.7				0.143
	195.3	0.082		0.210	
I	5		0.024		
	6.3				-0.003
	12.5	-0.019	0.074	0.051	
	15.6				0.016
	31.3	0.008	0.013	0.097	
	39.1				0.079
	78.1	0.069		0.146	
	97.7				0.108
	195.3	0.115		0.161	
J	5		0.072		
	6.3				0.036
	12.5	0.014	0.043	0.085	
	15.6				-0.010
	31.3	0.048	-0.044	0.046	
	39.1				-0.025
	78.1	0.032		0.052	
	97.7				-0.051
	195.3	-0.005		-0.015	

Figure A3. Phase II. Estimated difference in bias with serum samples. Dashed lines (red) indicate range of acceptable difference in bias for commutable samples.

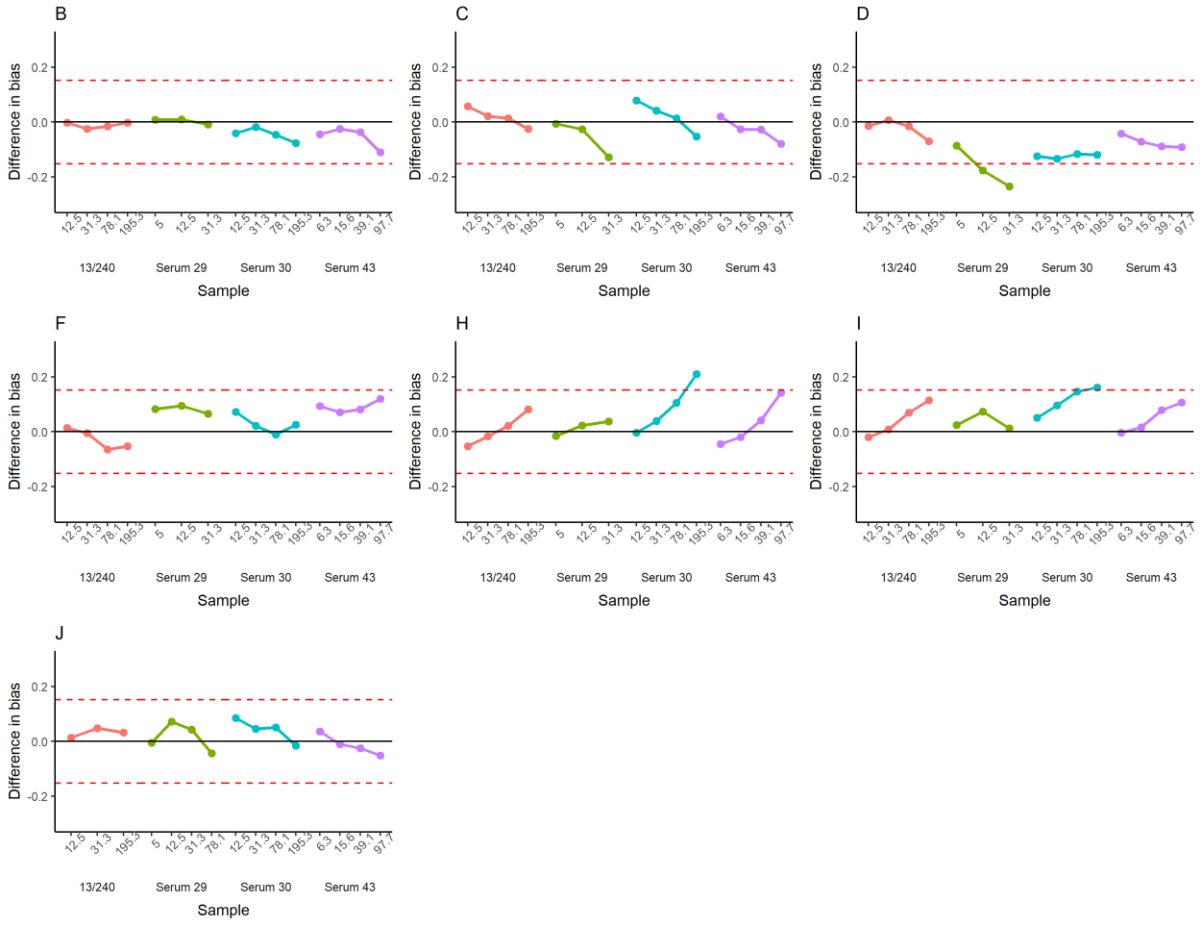

Figure A4. Phase II. Mean bias estimates for individual samples and dilutions. Dashed lines indicate mean method bias for serum samples (red lines) and range of acceptable values for other preparations demonstrating commutability (black lines).

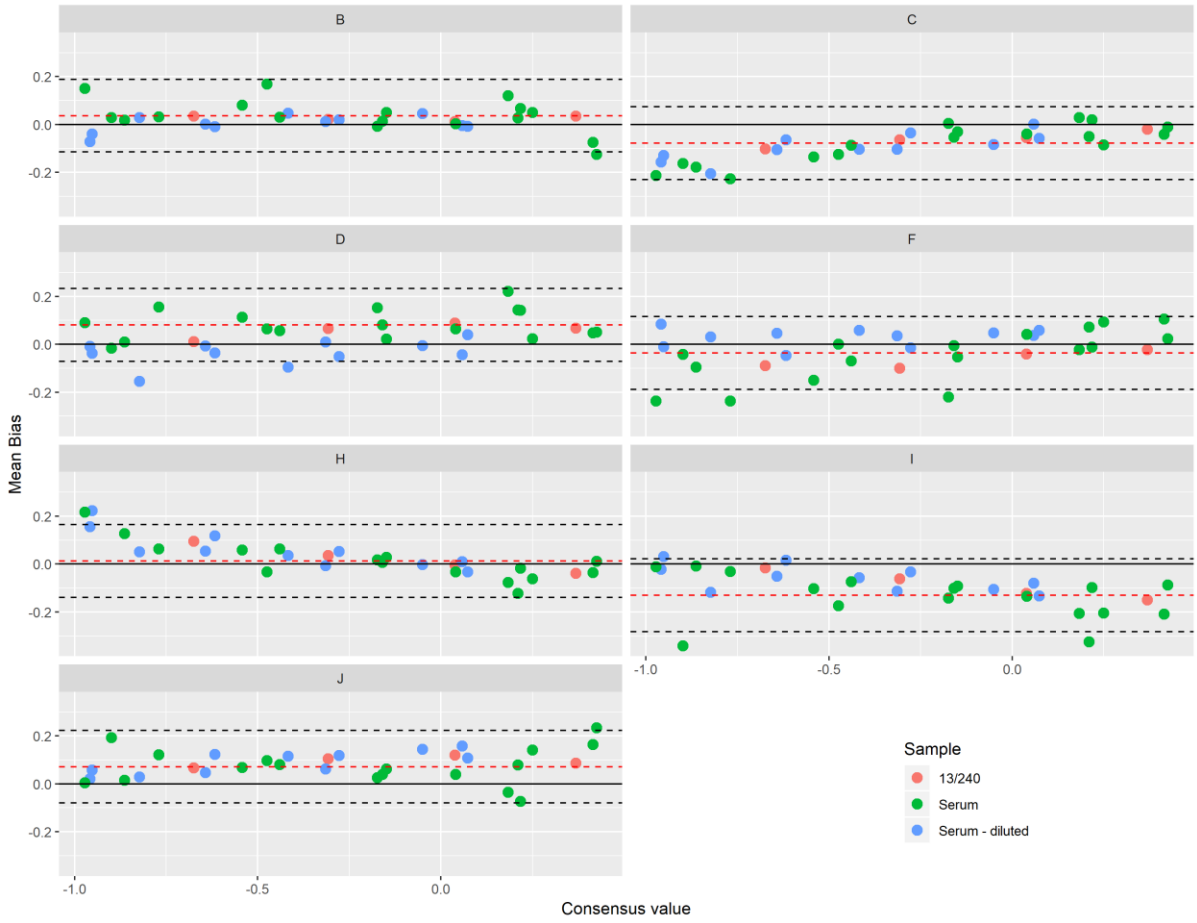

Table A7. Phase II. Fitted slopes for bias vs consensus values. Slopes >0.10 highlighted in red, slopes <-0.10 highlighted in blue.

Method Code	13/240	Serum 29	Serum 30	Serum 43
B	-0.003	0.022	0.040	0.058
C	0.074	0.160	0.126	0.088
D	0.056	0.192	-0.010	0.049
F	0.074	0.023	0.051	-0.024
H	-0.128	-0.069	-0.209	-0.180
I	-0.133	0.017	-0.113	-0.114
J	0.022	0.151	0.088	0.081

Table A8. Phase II. Inter-method variability in serum sample results

Serum sample	Inter-method %GCV	
	Reported	Rel. to 13/240
1	14.2	14.1
2	32.4	31.9
3	14.7	14.3
5	20.7	20.0
15	44.1	42.4
16	30.0	28.7
17	25.0	24.1
18	48.9	47.4
19	18.4	17.9
21	51.7	52.0
24	44.2	44.2
29	31.4	31.6
30	30.5	31.6
34	37.3	36.5
40	32.5	31.5
43	33.2	34.2
44	16.9	17.0

References

[1] CLSI. Characterization and Qualification of Commutable Reference Materials for Laboratory Medicine; Approved Guideline. CLSI document EP30-A. Wayne, PA: Clinical and Laboratory Standards Institute; 2010

[2] Mair, P., Schoenbrodt, F., & Wilcox, R (2017). WRS2: Wilcox robust estimation and testing.

Appendix B.
Draft Instructions for Use for 13/240

Medicines & Healthcare products Regulatory Agency

NIBSC Confidence in Biological Medicines

WHO International Standard
2nd International Standard for Tetanus Immunoglobulin Human
NIBSC code: 13/240
Instructions for use
(Version 2.00, Dated 04/08/2016)

1. INTENDED USE
This standard preparation of tetanus immunoglobulin human (coded 13/240) is intended for calibration of assays used to determine the potency of therapeutic tetanus immune globulin products. 13/240 is also suitable for calibration of immunoassays used to measure anti-tetanus antibody titres in human serum. It is the 2nd International Standard for Tetanus Immunoglobulin Human replacing the 1st IS coded TE-3. The 2nd IS (13/240) was adopted by the Expert Committee on Biological Standardization in October 2019.

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
13/240 has an assigned value of 45 IU/ampoule, based on the results of in vivo potency asays obtained in an International Collaborative Study [1].

4. CONTENTS
Country of origin of biological material: Croatia.
Each ampoule contains the freeze-dried residue of a 1 ml fill of purified human tetanus immunoglobulin

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

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

7. USE OF MATERIAL
No attempt should be made to weigh out any portion of the freeze-dried material prior to reconstitution

The entire contents of each ampoule should be completely resuspended in 1 ml of sterile ultrapure water

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.

The results of stability studies performed at NIBSC suggest that 13/240 will be very stable [1].

No data is available yet on stability after reconstitution. Therefore, once opened and reconstituted, users are advised to use the material on the same day.

9. REFERENCES
[1] Study report - to be cited after ECBG

10. ACKNOWLEDGEMENTS
Twenty laboratories contributed to the international collaborative study and all are acknowledged in the study report [1]. The donator of the source material used to develop the standard is also acknowledged in this report.

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

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

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

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

Physical and Chemical properties			
Physical appearance: Freeze-dried cake		Corrosive:	No
Stable:	Yes	Oxidising:	No
Hygroscopic:	No	Irritant:	No
Flammable:	No	Handling:See caution, Section 2	
Other (specify):	Contains material derived from human plasma		
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		

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WHO International Laboratory for Biological Standards,
UK Official Medicines Control Laboratory

World Health Organization

Medicines & Healthcare products Regulatory Agency

NIBSC Confidence in Biological Medicines

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: 0.073 g (mean dry weight of ampoule contents)
Toxicity Statement: Non-toxic
Veterinary certificate or other statement if applicable. **Attached**: No

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

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