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Collaborative study: Calibration of Replacement International Standard for Diphtheria Toxoid for use in Flocculation Test

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Summary

We report here the results of a study for the characterization of a preparation of diphtheria toxoid (coded Preparation A) and its calibration in flocculation (Lf) units. Calibration was performed using Ramon flocculation method, standardised using the 2nd International Standard (IS) for Diphtheria Toxoid for use in Flocculation Test (02/176, 1100 Lf/ampoule). Preparation A was found to have a unitage of 1874 Lf/ampoule based on results from 25 laboratories in 15 different countries. A second diphtheria toxoid preparation, coded Preparation B, was included in the same collaborative study and was found to have a unitage of 714 Lf/ampoule. There was good agreement in the results obtained (intra-laboratory and inter-laboratory) for both preparations.

Real time stability data shows the candidate material to be stable at the normal storage temperature of -20°C and suitable for use up to 1 month after reconstitution when stored at +4°C. Data from preliminary accelerated thermal degradation studies showed no temperature dependent loss in activity after storage for 1 month, indicating that the standard is likely to have good long-term stability. Further studies at later time points will be performed post establishment.

This study also provided an opportunity to assess the use of alternative methods for measuring Lf. Participants were asked to determine the Lf value of Preparation A and B using an ELISA assay established at NIBSC, or other suitable in-house methods for Lf determination. Ten laboratories participated by performing ELISA according to the NIBSC protocol, one laboratory performed flocculation using laser-light scattering according to an in-house protocol, and one laboratory performed another in-house ELISA method. Results suggest that these methods may provide suitable alternatives to the Ramon flocculation test, subject to validation, and that the replacement WHO IS could act as a suitable reference preparation for these methods.

Based on the results of this study and with the agreement of participants, it is proposed that preparation A be established as the 3rd WHO International Standard for Diphtheria Toxoid for use in Flocculation Test with an assigned value of 1870 Lf/ampoule.

Introduction

Diphtheria is caused by exotoxin-producing strains of the bacterium *Corynebacterium diphtheriae*. Active immunization against diphtheria is based on the use of diphtheria toxoid (DTxd), a chemically detoxified preparation of diphtheria toxin, to induce protective antibody responses. Diphtheria vaccines form an essential component of the primary immunization schedule of children and have been part of the WHO Expanded Programme on Immunization (together with tetanus and pertussis components) since its inception in 1974. The bulk toxoid intermediates of diphtheria vaccines can also be used as carrier proteins in polysaccharide conjugate vaccines against invasive bacterial infections caused by *N. meningitidis*, *H. influenzae* and *S. pneumonia*.

DTxd is produced by growing the toxin-producing *C. diphtheriae* in liquid media and converting the toxin to inactive toxoid by treatment with formaldehyde. Antigenic strength and purity of the bulk toxoid is evaluated by measurement of ‘limit of flocculation’ (Lf) units. DTxd for use in production of vaccines for human use must be shown to meet minimum requirements for purity (Lf units per milligram of protein nitrogen). The current WHO minimum requirement for antigenic purity of Dtxd has been set as not less than 1500 Lf/mg of protein nitrogen [1]. Measurement of antigen content in Lf also serves as a good indicator of the consistency of production, and testing of the crude toxin prior to inactivation is recommended for monitoring purposes.

The flocculation unit was originally a relative unit defined as the amount of toxin (or toxoid) equivalent to one International Unit (IU) of antitoxin in the flocculation test (Ramon version) [2]. Flocculation is determined using an *in vitro* method based on the observation that antigen and antibody aggregate and form visible floccules when mixed in certain proportions in solution. The precipitate develops more rapidly when equivalent amounts of antigen and antitoxin are present than when an excess of either is available. In the original Ramon flocculation method the antigen concentration is kept constant and different amounts of antitoxin are added to a series of tubes. The antitoxin content of the first tube to flocculate can be used to calculate the Lf value of the sample. The time in minutes for the first tube to flocculate is known as the Kf value, and is a useful indicator of the quality of the antigen and antitoxin used.

In 1970 the WHO Expert Committee on Biological Standardization decided to examine the feasibility of defining the Lf unit internationally by means of a reference toxoid preparation calibrated in Lf, rather than by means of an antitoxin preparation [3]. Subsequently the 1st International Reference Reagent of Diphtheria Toxoid for Flocculation Test (DIFT) was established in 1989, calibrated against the fifth International Reference Preparation of Diphtheria Antitoxin for the Flocculation Test (DIF), carrying the valid Lf definition at the time [4, 5]. DIFT was replaced with the 2nd International Standard (IS) for Diphtheria toxoid for use in Flocculation test (02/176) in 2007 following depletion of stocks [6].

Due to its simplicity, speed and economy, flocculation remains the primary method used by vaccine manufacturers to evaluate toxin and toxoid concentrations in Lf. Most laboratories use the toxoid IS to calibrate a suitable antitoxin in Lf-equivalent (Lf-eq) units. This antitoxin can then be used routinely to estimate Lf values of unknown toxin or toxoid samples. Various modifications of the original Ramon flocculation test exist, for example, keeping antitoxin concentration constant and adding different amounts of antigen (Dean-Webb method), or varying the concentrations of both components simultaneously (Levine-Wyman method). However different versions of the method give different equivalence amounts, therefore it is important that the same version of the test is used for calibration of the local reference antitoxin and for routine use [5].

Stocks of the 2nd IS (02/176) are in limited supply and a project was initiated to calibrate and establish a replacement standard. Candidate material for the replacement standard was provided to NIBSC for formulation and filling prior to freeze-drying. A similar material from another manufacturer was also provided to NIBSC, and prepared in the same way, for a proposed new Pharmacopoeial Reference Standard (RS) for Diphtheria Toxoid. Collaborative study (NIBSC code CS509) was initiated with the primary aim of calibrating these materials in Lf units using Ramon flocculation test standardised against the 2nd IS. 26 laboratories in 15 countries (Argentina, Belgium, Canada, China, Croatia, Cuba, Denmark, France, Hungary, India, Indonesia, Japan, Republic of Korea, The Netherlands and United Kingdom) participated in the collaborative study and 25 of these performed flocculation assays used for calibration of the candidate standards. A secondary aim of the collaborative study was to assess the suitability of alternative antigen detection methods for measuring Lf of diphtheria toxoid. 10 laboratories performed a capture ELISA assay developed at NIBSC. In addition 1 laboratory returned results from another in-house ELISA and 1 laboratory performed an alternative flocculation method using laser light scattering to obtain a more objective detection of antigen-antibody complexes.

The participating laboratories are listed in the Appendix and are referred to throughout this report by a code number, allocated at random, and not related to the order of listing.

Bulk material and processing

Bulk purified diphtheria toxoid (Lot D-70-01, ca. 1840 Lf/ml, 2141 Lf/mg PN) was kindly provided by Statens Serum Institut (SSI, Copenhagen, Denmark). Diluted bulk purified diphtheria toxoid (Lot 292202, ca. 750 Lf/ml, 2197 Lf/mg PN) was kindly provided by Serum Institute of India (SII, Pune, India). Both materials complied with the required quality control tests for bulk purified toxoids including safety, sterility and antigenic purity. The antigen content was confirmed by in-house capture ELISA at NIBSC. Five litres of diphtheria toxoid D-70-01 and seven litres of diphtheria toxoid 292202 was stabilized by the addition of 0.1 M sodium chloride and 1% trehalose before freeze-drying. Filling (1 ml per ampoule) was performed within NIBSC's Standard Processing Division on 21st November 2013 (SSI toxoid) and 15th May 2014 (SII toxoid) using a Bausch and Strobel Filling Machine (AFV5090). The material was stirred constantly during filling and the temperature was maintained between +4-8°C. The filled ampoules were freeze-dried using a Sorial CS100 freeze-dryer, with a 4 day cycle initiated on the day of filling. Ampoules were sealed after completion of the freeze-drying cycle and stored in the dark at -20°C. The finished products were coded 13/212 (SSI) and 14/132 (SII) and were labelled as Preparation A or Preparation B respectively for the collaborative study. A total of 5109 ampoules of 13/212 (preparation A) and 6810 ampoules of 14/132 (preparation B) were filled at NIBSC.

Characterization of freeze-dried candidate standards

After filling and freeze-drying, the candidate toxoids were examined for appearance, residual moisture content, oxygen head space and total antigen content (results are summarized in Table 1). The lyophilized product for both toxoids was of very good appearance, giving rise to robust and homogenous cakes. The precision of fill was determined by weighing ampoules after fill. Representative ampoules were weighed at 1 minute intervals throughout the production run. A total of 179 ampoules were weighed for 13/212 (preparation A) and the mean fill mass was 1.01 g with a coefficient of variation (CV) of 0.21%. For 14/132 (preparation B) a total of 232 ampoules were weighed and the mean fill mass was 1.01 g with a CV of 0.14%. Ampoules were sealed under boil-off gas from high purity liquid nitrogen (99.99%) and measurement of the mean oxygen head space after sealing served as a measure of ampoule integrity. The mean oxygen head space was measured non-invasively by frequency modulated spectroscopy (FMS 760, Lighthouse Instruments, Charlottesville, USA). Residual moisture content was measured using the coulometric Karl Fischer method in a dry box environment (Mitsubishi CA100, A1 Envirosciences, Cramlington, UK) with total moisture expressed as a percentage of the mean dry weight of the ampoule contents. The results of measurements of residual moisture content and oxygen headspace are summarized in Table 1. Both candidate preparations fulfil WHO requirements for reference preparations regarding precision of fill, residual moisture and oxygen head space.

The antigen content was determined by Ramon flocculation and in-house antigen ELISA against the 2nd WHO IS at NIBSC. For preparation A the antigen content was measured as 1847 Lf/ampoule by ELISA and 1840 Lf/ampoule by flocculation test. For preparation B the antigen content was measured as 686 Lf/ampoule by ELISA and 675 Lf/ampoule by flocculation test. Based on antigen estimates obtained by ELISA for the bulk materials, the recovery of antigen content after filling and freeze-drying was estimated to be approximately 95% (preparation A) and 92% (preparation B).

Collaborative study design and methods

Study design

Since the original definition of the Lf unit was the amount of toxin or toxoid equivalent in the Ramon version of the flocculation test with one unit of antitoxin, the calibration of the proposed toxoids is based on the Ramon version. Participants were provided with method guidelines based on established World Health Organisation (WHO) and European Pharmacopoeia (Ph Eur) methods [7, 8]. Participants were asked to use their routine in-house antitoxin for flocculation test, and to pre-calibrate this in Lf-eq units against the 2nd IS to ensure traceability of the Lf unit and to standardise flocculation results. A summary of antitoxins used in the study is given in Table 2. Information for performing the pre-calibration step was included in the method guidelines provided to all participants.

Each laboratory was provided with sufficient ampoules of the 2nd IS and of the candidate diphtheria toxoid standards to perform one assay to pre-calibrate their antitoxin, and four independent assays to calibrate the candidate materials using a new ampoule for every test. The samples were sent to participants with instructions for storage and use. Recommendations for suitable initial dilutions for Preparations A and B were provided to participants based on preliminary antigen estimates obtained at NIBSC.

Additional ampoules of the 2nd IS and of the candidate diphtheria toxoid standards were provided to some participants to perform an in-house ELISA developed at NIBSC for measuring Lf, or other suitable alternative method for Lf determination. For the NIBSC ELISA, participants were also provided with method guidelines and the critical antibody reagents used for capture and detection of the diphtheria toxoid (NIBSC 10/130 and 10/128 respectively). Participants were asked to perform three independent assays for each alternative method using the same ampoule for all replicate assays. Estimates of diphtheria toxoid in Lf were standardised against the 2nd IS.

Assay methods

Ramon flocculation assay:

For pre-calibration of the antitoxin, participants were recommended to prepare a series of seven tubes containing 35 IU to 65 IU of antitoxin and 50 Lf of the 2nd IS for diphtheria toxoid for use in flocculation test (02/176) in a total volume of 2 ml. Based on the 1st tube to flocculate (which would contain 50 Lf-eq units of antitoxin) they were asked to calculate the Lf-eq value of their original antitoxin sample, taking any initial dilution factor into account, and to use this Lf-eq value for the subsequent assays for calibration of the candidate diphtheria toxoid standards.

For calibration of the candidate standards it was recommended to initiate the assay with a series of seven tubes containing 35 Lf-eq units to 65 Lf-eq units of diphtheria antitoxin, and diphtheria toxoid to the assumed amount of 50 Lf, in a total volume of 2 ml. Participants were asked to record the first, second and third mixtures to flocculate as well as the time taken for the first flocculation to appear. The first tube in which flocculation appeared was used to calculate the Lf value of the toxoid sample. Participants could refine their dilution series after the first assay using a narrower range (e.g. 2 Lf increases from tube to tube) to obtain a more precise estimate of Lf.

If flocculation appeared first in the first or last tube of the series the test had to be repeated using either a different range of the antitoxin or different dilution of test toxoid.

NIBSC Enzyme Linked Immunosorbent Assay (ELISA):

A capture ELISA has been developed at NIBSC to monitor the diphtheria antigen content and degree of adsorption in final vaccine products [9]. The assay is in routine use as a consistency assay and also serves as the identity test for diphtheria antigen. The method was performed as described previously [9], with the exception of using recently prepared freeze-dried stocks of the capture and detection antibodies (reconstituted in 0.5ml H_2O and diluted 1/200 for use in the assay). The reference toxoid (02/176) and the candidate diphtheria toxoid standards were titrated in the range of approximately 0.055-0.0004 Lf/ml for the assay.

Other methods:

Flocculation assay by laser light-scattering [7, 10] was performed using a platelet aggregometer. The assay was performed with a series of three or four tubes containing 6.25 Lf-eq units to 12.5 Lf-eq units of diphtheria antitoxin, and diphtheria toxoid to the assumed amount of 10 Lf, in a total volume of 400 µl. The time taken to acquire particle counts of 50,000 was recorded and the data analysed using quadratic regression (log antitoxin concentration vs. log time). Lf values were calculated as the local minimum value of the regression curve.

Another in-house antigen ELISA assay was also performed. Plates were coated with diphtheria antitoxin (ST-ADS-36-07) diluted to 1 Lf-eq/ml. Following blocking with PBS + 0.5% BSA, samples and reference toxoid (02/176) were titrated in the range of approximately 0.5-0.004 Lf/ml. Bound toxoid was detected with a horse anti-diphtheria peroxidase conjugated antibody followed by substrate. Data was analysed using a 4-parameter fit standard curve (log dose vs. response) with concentrations (Lf/ampoule) of toxoids A and B calculated from interpolated values.

Reporting of data and statistical analysis

All raw data together with assay details were returned to NIBSC (using provided data sheets) to permit independent analysis. Any deviation from the method guidelines was reported to NIBSC.

Estimates from NIBSC ELISA for toxoids A and B were calculated relative to IS 02/176 by parallel-line analysis using CombiStats [11]. A log transformation was applied to the assay responses and dilutions on a linear section used for analysis. Linearity and parallelism was assessed by analysis of variance with deviations from linearity and parallelism considered significant at the 1% level ($p<0.01$).

Results from all valid assays were combined as unweighted geometric means (GM) for each laboratory and these laboratory means were used to calculate overall unweighted geometric means. Variability between assays and laboratories has been expressed using geometric coefficients of variation (GCV = {10s-1}×100% where s is the standard deviation of the $\log_{10}$ transformed estimates). Comparisons between flocculation and NIBSC ELISA assays have been made by unpaired t-test of log transformed results.

Stability studies

To determine the stability of the candidate toxoids an accelerated degradation study and real time monitoring of the material was initiated at NIBSC.

For the accelerated degradation study, representative samples (ampoules) for Preparation A and B were stored at +4, +20, +37, +45 and +56°C in addition to the recommended storage temperature of -20°C. Results from the collaborative study to establish the 2nd WHO IS for diphtheria and tetanus toxoid for flocculation test suggest that, once freeze-dried, these materials are likely to be highly stable [12]. Preliminary data was obtained by testing samples after 1 month of storage with further data to be collected at later time points (up to 4 years).

The real time stability of the freeze dried material was assessed by comparing Lf values obtained for ampoules stored at -20°C over time, with data available up to 18 months for preparation A and 12 months for preparation B. Stability of the reconstituted material was also assessed by comparing Lf values obtained for ampoules reconstituted and stored at +4°C for one month with values for ampoules reconstituted on the day of the assay. The samples were assessed using the Ramon flocculation test and NIBSC ELISA as described previously.

Results

Results contributed to the study

Flocculation assay results were returned by 25 laboratories and all performed four independent assays of toxoids A and B, with the exception of lab 3 where three assays were performed. The four independent assays performed by lab 23 used a pooled sample of the reconstituted diphtheria toxoids A and B rather than a new ampoule for every test. One assay for each toxoid was excluded from the results for lab 23 as the first and second tubes to flocculate were more than one tube apart.

Several laboratories (lab codes 7, 12, 14, 16 and 18) chose to perform two tests with each reconstituted ampoule of the candidate diphtheria toxoid standards; one ‘broad’ range according to the dilution range suggested in the method guidelines, followed by one ‘narrow’ range using smaller increases from tube to tube to obtain a more precise estimate of Lf. Lab 9 performed a ‘broad’ range and a ‘narrow’ range test for the first assay and then ‘narrow’ range tests for the following three independent assays. Two replicate tests were performed by lab 24 with each reconstituted ampoule.

NIBSC ELISA results were returned by 10 laboratories and all performed three assays except for lab 23 where four assays were performed. No results were calculated for lab 25 due to high residual error and poor model fit.

Results from flocculation assay by laser light-scattering were returned by one laboratory. Three independent assays were performed, with assays 2 and 3 using a different ampoule of the candidate diphtheria toxoids to assay 1. One laboratory returned results of three independent assays from their own in-house ELISA assay.

Flocculation assay results

Tables 3 and 4 summarise the results (Lf/ampoule) obtained for toxoids A and B in Ramon flocculation assays. Where a laboratory performed both ‘broad’ and ‘narrow’ dilution ranges, the results obtained using the ‘narrow’ range have been used. Where replicate tests were performed the geometric mean of the results was taken. An overall geometric mean of 1874 Lf/ampoule (95% confidence limits: 1839-1910; GCV 4.7%; n=25) was determined for toxoid A and 714 Lf/ampoule

(95% confidence limits: 691-738; GCV 8.2%; n=25) for toxoid B. Within-laboratory GCV's ranged from 0% (all assays giving the same result) to 7% with the exception of toxoid A in lab 1.

Using the "broad" range results from labs 7, 12, 14, 16 and 18 gave similar overall means of 1870 Lf/ampoule and 716 Lf/ampoule for toxoids A and B respectively.

The time taken for the first flocculation to occur, known as Kf, varied dramatically depending on the antitoxin preparation used, and was noticeably long for antitoxins 63/007 (NIBSC) and 280571 (SSI). A summary of average Kf times observed for toxoids A and B for each antitoxin is shown in table 2. The average Kf ranged from 4 minutes to 74 minutes for toxoid A and from 8 minutes to 167 minutes for toxoid B. In all cases Kf times were longer for toxoid B than for toxoid A. The Kf values observed for toxoid A are comparable to those obtained with the 2nd IS when determining the Lf-eq value of the antitoxin preparation (Table 2).

NIBSC ELISA results

Tables 5 and 6 summarise the results (Lf/ampoule) obtained for toxoids A and B in NIBSC ELISA assays. With the exception of lab 25, valid results were obtained in all cases. An overall geometric mean of 1806 Lf/ampoule (95% confidence limits: 1744-1870; GCV 4.6%; n=9) was determined for toxoid A and 675 Lf/ampoule (95% confidence limits: 647-705; GCV 5.8%; n=9) for toxoid B. The result from lab 22 was found to be an outlier for toxoid A ($p<0.05$ in Grubbs' test on log laboratory means) and results calculated excluding this lab were 1830 Lf/ampoule (95% confidence limits: 1791-1869; GCV 2.6%; n=8) for toxoid A and 686 Lf/ampoule (95% confidence limits: 667-706; GCV 3.5%; n=8) for toxoid B. Within-laboratory GCV's ranged from 0.3% to 16.3%.

No significant difference between flocculation and antigen results was detected for toxoid A (p=0.209; lab 22 excluded) or toxoid B (p=0.170; lab 22 excluded).

Other methods

For flocculation assay by laser light scattering, geometric mean values of 1903 Lf/ampoule and 721 Lf/ampoule were determined for toxoid A and B respectively. The within-laboratory GCV was 0.8% for toxoid A and 2.5% for toxoid B. For the other in-house antigen ELISA, geometric mean values of 1920 Lf/ampoule and 723 Lf/ampoule were determined for toxoid A and B respectively. The within-laboratory GCV was 5.1% for toxoid A and 5.6% for toxoid B.

Laboratory means for each toxoid and all assay types are shown in Figures 1 and 2.

Stability

Preliminary data (1 month) from accelerated degradation studies showed no change in Lf values obtained for toxoids A or B over the range of temperatures used and therefore no prediction of stability could be made. This suggests that the preparations will be highly stable when stored at the recommended storage temperature of -20°C, however further studies will be performed at later time points to try and obtain a prediction of the rate of loss of activity.

Table 7 summarises the results (Lf/ampoule) obtained in Ramon flocculation assay in real time for the two toxoid preparations. Results for ampoules stored at the normal storage temperature (-20°C) were consistent during the period of assessment (18 months for toxoid A and 12 months for toxoid B). Comparable results were also obtained for ampoules reconstituted and stored at +4°C for one

month. All samples were also tested by NIBSC ELISA and similar results were obtained (not shown).

Conclusions

It is proposed to recommend sample coded A (13/212) as suitable replacement WHO International Standard for Diphtheria toxoid for use in flocculation test. It is recommended to assign a value of 1870 Lf/ampoule (rounded from 1874 Lf/ampoule) based on flocculation results returned from 25 laboratories. This proposal was agreed by the participating laboratories. NIBSC will act as custodian of the standard which will be stored under assured temperature controlled conditions within the Institute's Centre for Biological Reference Materials, at the address listed in the introduction. The second toxoid included in the study, coded B, is a candidate Pharmacopoeial Reference Standard.

Ampoules coded 13/212 were tested and confirmed to fully comply with WHO recommendations for precision of fill, residual moisture content and integrity. Preliminary data from accelerated degradation studies indicates that the proposed standard will have adequate long-term stability and real-time monitoring shows no loss in activity (Lf content) to date. Stability will continue to be monitored at NIBSC and will include further measurements of Lf in samples stored at elevated temperature for extended periods (up to 4 years from the date of storage at elevated temperature).

A total of 5109 ampoules of 13/212 were filled at NIBSC. After collaborative study, in-house measurements and accelerated degradation studies, 4800 ampoules remain available at NIBSC (-20°C) for use as the WHO IS. Based on current use, it can be predicted that this will be sufficient for the next 15-20 years.

Comparable results to the original Ramon flocculation test were obtained when using a laser light-scattering platelet aggregometer as the detection system for flocculation. Similarly, Lf results returned by ELISA methods were not significantly different to flocculation, and suggest ELISA may be used as a suitable alternative to flocculation test for measuring Lf of diphtheria toxoid samples, subject to validation. Based on the performance of the candidate toxoids in this study, they could act as suitable reference preparations in ELISA methods for Lf determination.

Recommendation

Based on the results of Ramon flocculation assay performed in the collaborative study, the material coded preparation A can be recommended as the 3rd WHO IS for Diphtheria Toxoid for use in Flocculation Test. The recommended unitage assigned to preparation A is proposed as 1870 Lf/ampoule based on flocculation assay results returned by 25 laboratories.

Comments from participants

All 26 participants were sent a draft report and asked to comment on the content and conclusions, and to confirm that their results had been reported correctly. Thirteen participants responded (50%) and twelve confirmed that they agreed with the content and recommendations. Data had been reported incorrectly for one participant (lab 8) for candidate A. The mistake did not change the final recommendation and this lab agreed with the content and conclusions after correction of their data.

Comments and follow-up questions regarding the study are summarised below

1. Lab 25 suggested that a brief conclusion be drawn regarding the use of ELISA for Lf determination. This has been added to the report.
2. Lab 22 asked for future assistance if they were to establish NIBSC ELISA method to help ensure accuracy of their results. The result for candidate A was considered as an outlier in this study, however it was noted that their antigen estimate obtained by ELISA was very close to another labs result for flocculation, and that it may not have been an outlier if there were more results for this method.
3. Lab 25 asked for further clarification on the cause of the invalidity for their NIBSC ELISA results and a response was provided to them.

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Table 1. Summary of stabilised, freeze-dried candidate standards

	Preparation A	**Preparation B**
Toxoid Manufacturer	Statens Serum Institut	Serum Institute of India
NIBSC Code	13/212	14/132
No. Ampoules filled	5109	6810
Appearance	Robust homogenous cake	Robust homogenous cake
Mean fill mass	1.01 g (CV 0.21%) (*n*=179)	1.01 g (CV 0.14%) (*n*=232)
Mean dry weight	0.03 g (CV 0.62%) (*n*=6)	0.03 g (CV 2.04%) (*n*=6)
Mean residual moisture	0.38% (CV 15.27) (*n*=12)	0.58% (CV 21.70) (*n*=12)
Mean oxygen head space	0.64% (CV 16.72) (*n*=12)	0.30% (CV 36.06) (*n*=12)

Table 2. Summary of antitoxins used by participants for flocculation test

Supplier	**Batch**	**No. of labs**	**Lf-eq units/ml**	**Average Kf time, min**		
				2nd IS 02/176	**A 13/212**	**B 14/132**
Bilthoven Biologicals B.V.	515/3	1	200	15	12	27
Biological E Ltd	DAT/02/2014	1	104	5	4	12
Central Drugs Laboratory	01/11-DATF	1	100	18	8	10
	DATF/0114	1	100	4	4	8
Institut of Immunology	207	1	3330	10	8	16
	209	1	3429	12	8	20
Institute Finlay	ADRF (10)/10	1	1400	5	5	8
National Institutes for food and drug Control	0048	1	1000	13	11	25
National Institute of Infectious Diseases	Lot 4	6	1109* (1100-1280)	7 (3-9)	7 (3-9)	12 (4-15)
NIBSC	63/007	5	619** (542 – 742)	56 (51-59)	74 (63-83)	167 (145-191)
PT. Bio Farma	ST-ADS-36-07	2	2055 (1909-2200)	11 (8-14)	14 (10-19)	25 (16-35)
Sanofi Pasteur	S3181	1	1200	18	8	21
Serum Institute of India	ADS 1/2008	1	1200	5	4	8
Statens Serum Institut	280571 Lot: 10829-03-0001	1	120[a]	53[a]	41[b]	81[b]
	280571 Lot: 10829-03-0002	1	113[a]	51[a]	39[c]	76[c]
ViNS Bioproducts LTD	04AD13001	1	1000	7	8	9

*when reconstituted in 1 ml, **when reconstituted in 2 ml

Where more than one participant used the same antitoxin the average result taken across labs is shown with the range of lab means indicated in brackets. Individual Lf-eq units and Kf times with 02/176 are from 1 assay with one exception.[a] Individual Kf times for candidate diphtheria toxoids 13/212 and 14/132 are the mean of 4 assays with two exceptions.[b,c]

[a] Mean of 4 assays, [b] 1 assay, [c] Mean of 3 assays

Table 3. Flocculation assay results (Lf/ampoule) for Toxoid A

Lab	Assay 1	Assay 2	Assay 3	Assay 4	GM	GCV
1	1944	1831	1627	2237	1897	14.2%
2	1980	1980	1980	1980	1980	0%
3	1800	1980	1944	.	1906	5.2%
4	1980	1980	1980	1980	1980	0%
5	1800	1800	1800	1800	1800	0%
6	1980	1908	1908	1908	1926	1.9%
7*	1800	1800	1800	1872	1818	2.0%
8	1900	1900	1900	1900	1900	0%
9	1908	1908	1908	1908	1908	0%
10	1800	2000	1800	1960	1888	5.7%
11	1800	1980	1800	1800	1843	4.9%
12*	2016	2088	2016	2088	2052	2.0%
13	1800	1800	1800	1800	1800	0%
14*	1797	1797	1797	1797	1797	0%
16*	1800	1872	1872	1800	1836	2.3%
17	1800	1800	1800	1980	1843	4.9%
18*	1800	1800	1872	1800	1818	2.0%
19	1980	1908	1944	1980	1953	1.8%
20	1800	1800	1980	1980	1888	5.7%
21	1719	1891	1719	1719	1760	4.9%
22	1980	1980	1980	1980	1980	0%
23**	1620	1710	.	1620	1649	3.2%
24	1800	1980	1980	1888	1910	4.7%
25	1980	1980	1800	1980	1933	4.9%
27	1800	1800	1872	1872	1836	2.3%
Overall GM					1874	
95% Confidence Limits					1839 – 1910	
GCV					4.7%	

* Results from "narrow range" used
** Assay 3 excluded

Table 4. Flocculation assay results (Lf/ampoule) for Toxoid B

Lab	Assay 1	Assay 2	Assay 3	Assay 4	GM	GCV
1	720	720	678	763	720	4.9%
2	645	645	645	645	645	0%
3	.	675	705	735	705	4.4%
4	750	750	750	750	750	0%
5	675	675	675	675	675	0%
6	705	705	675	705	697	2.2%
7*	720	690	720	720	712	2.2%
8	750	750	750	750	750	0%
9	750	750	750	750	750	0%
10	750	750	750	717	742	2.3%
11	750	750	750	750	750	0%
12*	900	870	870	870	877	1.7%
13	600	600	600	600	600	0%
14*	671	671	671	671	671	0%
16*	720	750	750	750	742	2.1%
17	825	825	750	750	787	5.7%
18*	690	690	690	690	690	0%
19	750	750	795	750	761	3.0%
20	750	750	750	750	750	0%
21	645	645	645	645	645	0%
22	750	750	750	750	750	0%
23**	675	.	675	600	649	7.0%
24	675	712	675	675	684	2.7%
25	675	750	750	750	731	5.4%
27	675	675	675	675	675	0%
Overall GM					714	
95% Confidence Limits					691 - 738	
GCV					8.2%	

* Results from "narrow range" used
** Assay 2 excluded

Table 5. NIBSC ELISA assay results (Lf/ampoule) for Toxoid A

Lab	Assay 1	Assay 2	Assay 3	Assay 4	GM	GCV
16	1746	1840	1866	.	1817	3.6%
18	1705	1969	1778	.	1814	7.7%
19	1882	1776	1929	.	1862	4.3%
20	1657	1894	1805	.	1783	7.0%
21	2056	1924	1731	.	1899	9.1%
22	1687	1652	1549	.	1628	4.5%
23	1748	1698	1865	1854	1790	4.7%
25	.	.	.	.	.	.
26	1793	1817	2065	.	1888	8.1%
27	1720	1927	1728	.	1789	6.6%
Overall GM 95% Confidence Limits GCV					1806 1744 - 1870 4.6%	
Overall GM (excluding lab 22) 95% Confidence Limits GCV					1830 1791 - 1869 2.6%	

Table 6. NIBSC ELISA assay results (Lf/ampoule) for Toxoid B

Lab	Assay 1	Assay 2	Assay 3	Assay 4	GM	GCV
16	641	639	643	.	641	0.3%
18	800	667	663	.	707	11.3%
19	836	626	668	.	705	16.3%
20	660	748	658	.	688	7.6%
21	796	683	654	.	708	10.9%
22	636	607	552	.	597	7.4%
23	650	675	687	673	671	2.3%
25	.	.	.	.	.	.
26	658	655	773	.	693	9.9%
27	711	691	630	.	676	6.6%
Overall GM 95% Confidence Limits GCV					675 647 - 705 5.8%	
Overall GM (excluding lab 22) 95% Confidence Limits GCV					686 667 - 706 3.5%	

Figure 1. Results (Lf/ampoule) for Toxoid A

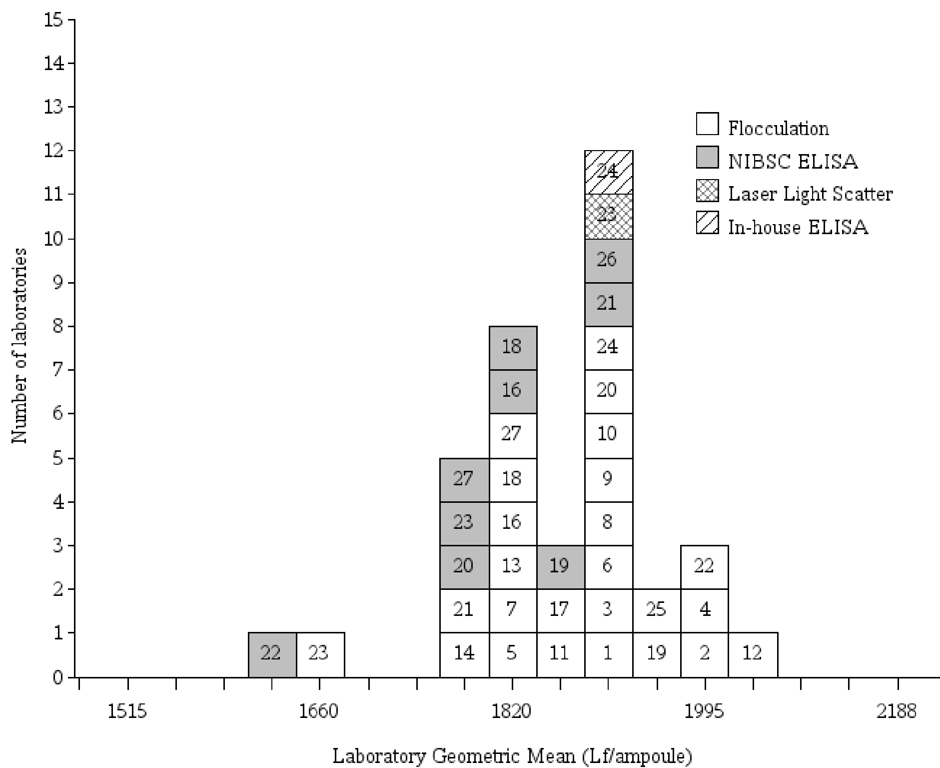

Figure 2. Results (Lf/ampoule) for Toxoid B

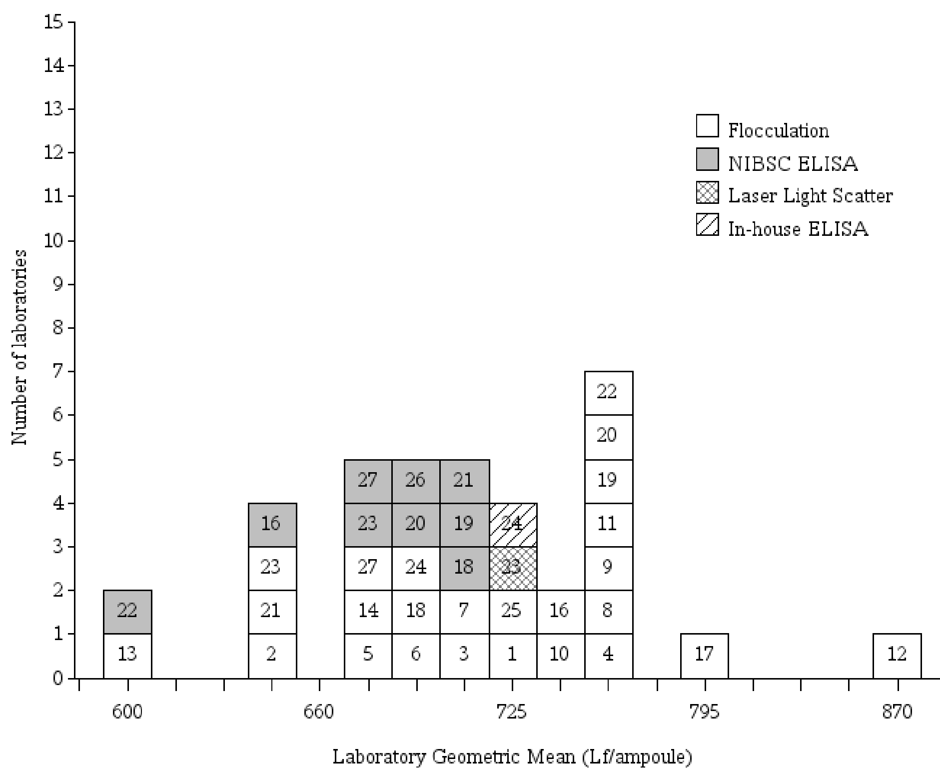

Table 7. Flocculation assay results (Lf/ampoule) obtained in real-time for stability monitoring

Reference point	Storage time	Toxoid A	Toxoid B
Post-fill (-20°C)*	2 weeks	-	675
	3 months	1840	675 675 675 675
	9 months	1800 1800 1872 1872	-
	12 months	-	675 675 645
	18 months	1800 1800 1800	-
Post-reconstitution (+4 °C)	1 month	1800 1872 1800	675 675 675

*Ampoules reconstituted on the day of the assay

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WHO International Standard
3rd International Standard for Diphtheria Toxoid for use in Flocculation Test
NIBSC code: 13/212
Instructions for use
(Version 2.00, Dated 12/05/2015)

1. INTENDED USE
This material has been prepared as a replacement for the 2nd International Standard for Diphtheria Toxoid for use in Flocculation Test (02/176). The replacement material coded 13/212 was established as the 3rd International Standard for standardisation of flocculation assays used to determine the Lf content of diphtheria toxoid.

13/212 may also be suitable as a reference preparation in other methods used to measure the Lf content of diphtheria toxoid, such as ELISA or SRD.

2. CAUTION
This preparation is not for administration to humans.

The material is not of human or bovine origin. 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 assigned unitage for 13/212 is 1870 Lf per ampoule. This is based on the results of flocculation tests performed by 25 laboratories in 15 different countries [1].

4. CONTENTS
Country of origin of biological material: Denmark.
Bulk purified diphtheria toxoid was kindly donated by Statens Serum Institut (Copenhagen). The bulk toxoid, 2141 Lf/mg protein nitrogen, was stabilised by the addition of sodium chloride (final concentration 0.1 M) and trehalose (final concentration 1% w/v). Filling and freeze-drying was performed at NIBSC in November 2013). The average dry weight of the ampoule contents is 0.03 g (CV 0.62%) and the mean residual moisture content was determined as 0.38% (CV 15.27%).

5. STORAGE
Store in the dark at -20°C
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 one ampoule should be completely re-suspended in 1 ml of a suitable solution (saline is suitable).

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.

It is the policy of WHO not to assign an expiry date to their international Reference Materials.
Preliminary Accelerated degradation studies performed [1] suggest that this material will be suitably stable when stored at the recommended storage temperature of -20°C, and the assigned unitage remains valid until the material is withdrawn or replaced.

Once reconstituted, 13/212 has been confirmed to be stable for up to 1 month following storage at +4°C [1].

9. REFERENCES
1. Coombes L, Rigsby P, Sesardic D, Stickings P. Collaborative Study for the Calibration of Replacement International Standard for Diphtheria Toxoid for use in Flocculation Test.

10. ACKNOWLEDGEMENTS
Statens Serum Institut is gratefully acknowledged for donation of the purified toxoid material used in the preparation of the replacement standard. All participants of the collaborative study performed to calibrate this replacement standard are gratefully acknowledged.

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/products/biological_reference_materials/frequently_asked_questions/how_are_international_units.aspx
Ordering standards from NIBSC:
http://www.nibsc.org/products/ordering_information/frequently_asked_questions.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

Physical and Chemical properties			
Physical appearance: Freeze-dried toxoid		Corrosive:	No
Stable:	Yes	Oxidising:	No
Hygroscopic:	No	Irritant:	No
Flammable:	No	Handling: See caution, Section 2	
Other (specify):	Contains material of bacterial origin		

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

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

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

15. LIABILITY AND LOSS

In the event that this document is translated into another language, the English language version shall prevail in the event of any inconsistencies between the documents.

Unless expressly stated otherwise by NIBSC, NIBSC's Standard Terms and Conditions for the Supply of Materials (available at http://www.nibsc.org/About_Us/Terms_and_Conditions.aspx or upon request by the Recipient) ("Conditions") apply to the exclusion of all other terms and are hereby incorporated into this document by reference. The Recipient's attention is drawn in particular to the provisions of clause 11 of the Conditions.

16. INFORMATION FOR CUSTOMS USE ONLY

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