EXPERT COMMITTEE ON BIOLOGICAL STANDARDIZATION

Geneva, 18 October to 21 October 2021

Collaborative study: Calibration of the 2nd WHO International Standard for Diphtheria Antitoxin Equine (DI)

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³Details of collaborative study participants are listed in the appendix to this report

NOTE:

This document has been prepared for the purpose of inviting comments and suggestions on the proposals contained therein, which will then be considered by the Expert Committee on Biological Standardization (ECBS). Comments MUST be received by 17 September 2021 and should be addressed to the World Health Organization, 1211 Geneva 27, Switzerland, attention: Technical Standards and Specifications (TSS). Comments may also be submitted electronically to the Responsible Officer: Dr Ivana Knezevic at email: knezevici@who.int.

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Summary

We report here the details of the preparation and characterization of the proposed 2nd International Standard for Diphtheria Antitoxin Equine (NIBSC code 18/180) and its calibration in International Units (IU). Calibration was performed by toxin neutralization test *in vivo* and *in vitro* (Vero cell assay), and potency was expressed relative to the 1st International Standard (DI, 19/236). A total of 14 participants from 9 different countries performed toxin neutralisation assays and returned data that was used to assign units to the proposed new standard. On additional participant performed an in-house immunoassay and returned results for comparison purposes only. Results obtained in the toxin neutralisation assays suggest that 18/180 has a diphtheria antitoxin potency of 57 International Units (IU) per ampoule.

Data from accelerated thermal degradation studies predicted there to be no significant loss in activity per year when stored at -20°C indicating that the standard is likely to have very good long-term stability. Real time stability data suggests that the candidate material will be suitable for use up to 1 year after reconstitution when stored at +2 - 8°C.

Based on the results of this study it is proposed that 18/180 be established as the 2nd WHO International Standard for Diphtheria Antitoxin Equine with an assigned potency of 57 IU/ampoule.

Introduction

The 1st International Standard for Diphtheria Antitoxin Equine (referred to as DI) was prepared in Copenhagen in 1934 and consists of a preparation of dried hyperimmune horse serum in ampoules. The IU for diphtheria antitoxin is defined by this standard as the activity contained in 0.0628 mg of the dried serum. To preserve stocks of the original dried serum from 1934, a new batch of the liquid standard is prepared by NIBSC approximately every 2 years as a solution of the dried serum in 66% v/v glycerol/saline, with a diphtheria antitoxin concentration of 10 IU/ml. The stock of the original dried serum is now exhausted, and it is proposed to replace this with a freeze-dried standard to provide a single homogenous batch that can be in use for several years. The most recent liquid batch of the 1st IS, NIBSC code 19/236, was filled on 04 December 2019 and was used to calibrate the proposed 2nd IS.

Diphtheria antitoxin (DAT) products produced from equine serum are essential medicines used for diphtheria therapy and outbreak management, and prophylaxis against suspected
cases of diphtheria in countries where disease is endemic. In countries with good vaccination coverage, DAT is stockpiled for emergency use. DAT must be shown to meet minimum requirements for potency [1] and an equine DAT standard calibrated in IU is essential to demonstrate that these requirements can be met. The potency assay described in the European Pharmacopoeia monograph for Diphtheria Antitoxin is a toxin neutralisation test (TNT) performed in guinea pigs or rabbits using intradermal challenge and a non-lethal end point [1]. In other regulatory jurisdictions, a subcutaneous challenge model with a lethal end point may be used (typically using guinea pigs). Both of these assay models (i.e. intradermal and subcutaneous challenge) were used by participants in this collaborative study.

The in vivo TNT can be performed at different toxin dose levels. For intradermal challenge the toxin dose level is referred to as the Lr dose, and include Lr/1, Lr/10, Lr/100 and Lr/1000. These toxin dose levels are defined as the smallest amount of diphtheria toxin which is capable of producing a positive erythematous reaction when mixed with 1, 0.1, 0.01 or 0.001 IU of diphtheria antitoxin respectively. The method described in the European Pharmacopoeia is performed at the Lr/100 toxin dose level. For subcutaneous challenge assays the dose levels are referred to as L/+1, L/+10, L/+100, L/+1000, following the principle described above but with the lethal end point.

As an alternative to the in vivo TNT, potency of DAT can be determined in vitro, using Vero cells which are susceptible to diphtheria toxin. The principle of the Vero cell assay is comparable to the in vivo assay with the exception that cytotoxicity in cultured Vero cells is used to determine assay end points. A quantitative method of titrating diphtheria toxin and antitoxin using Vero cells was first established in the 1950s [2] and this was developed into a microplate assay in the 1970s [3]. Assay end points can be determined visually (either by microscopy or by colour changes in the culture medium as an indicator of cell viability) or by the inclusion of tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and spectrophotometric analysis to determine cytotoxic end points [4]. Both of these approaches to determine assay end points were used by participants in this study. As with in vivo assays, the Vero cell TNT can be performed at different toxin dose levels (for example: Lcd/1, Lcd/10, Lcd/100 and Lcd/1000) where Lcd refers to the ‘Limit of cytopathic dose’. The Vero cell TNT can also be performed at a toxin dose level that is related to the absolute sensitivity of the cells to diphtheria toxin (referred to as MCD, Minimum Cytopathic Dose, or just CD).

A previous collaborative study that was performed to calibrate the 1st International Standard for Diphtheria Antitoxin Human (NIBSC product code 10/262) demonstrated good agreement between the potency estimates obtained for the candidate standard in both the in vivo and in vitro assay models [5].

For this new study, participating laboratories were invited to participate based on their ability to perform in vivo and/or in vitro TNT assays to determine the potency of diphtheria antitoxin. A total of 14 participants, consisting of DAT manufacturers and national control/regulatory laboratories from 9 different countries, were recruited to the study and returned data. 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. In addition, one laboratory performed an immunoassay and returned data for information and comparison purposes only.
**Bulk material and processing**

Refined diphtheria immunoglobulin prepared from horse serum was obtained from KM Biologics, formerly known as the Chem-Sero-Therapeutic Research Institute, Kaketsuken (Lot 0801, ca 3400 IU/ml, protein concentration 61.2 mg/ml). The source material was tested for diphtheria antitoxin potency at NIBSC using the Vero cell TNT with a toxin dose level of Lcd/100. Two separate assays were performed with the potency estimated in IU/ml relative to the 1st IS for Diphtheria Antitoxin Equine (19/236). Results were comparable to the labelled potency with a geometric mean estimate of 2622 IU/ml (individual assay estimates of 2022 IU/ml and 3400 IU/ml). The bulk material was diluted in phosphate buffered saline (PBS, pH 7.4) to approximately 100 IU/ml (based on labelled value) prior to freeze-drying. The need for additional stabilization of the diluted bulk material for freeze-drying was assessed using trial fills that were prepared with and without 1% trehalose as stabilizer. The freeze-dried product was of good appearance and stability was confirmed (by accelerated degradation study) to be satisfactory for both formulations (not shown). As a result, the definitive fill was prepared using bulk material diluted in PBS without any additional stabilizer. The absence of need for additional stabilization was most likely due to the high protein content of the bulk material.

Pooling of the bulk material was performed in a Class II microbiological safety cabinet with aseptic technique to maintain sterility. Filling (1 ml per ampoule) was performed within NIBSC’s Standard Processing Division on 26th October 2018 using a Bausch and Strobel Filling Machine (AVF5090) at ambient temperature. The filled ampoules were freeze-dried using a Serva CS15 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 product was coded 18/180.

**Characterization of freeze-dried product 18/180**

The lyophilized product was of very good appearance, giving rise to a robust and homogenous cake. The precision of fill was determined by weighing ampoules from across the production run after fill. A total of 111 ampoules were weighed and the mean fill mass was 1.007 g with a coefficient of variation (CV) of 0.26%. 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 Envirosiences, Cramlington, UK) with total moisture expressed as a percentage of the mean dry weight of the ampoule contents. The candidate preparation met, or was very close to meeting the criteria that is expected to be fulfilled by the vast majority of WHO biological reference materials [6]; Residual moisture and oxygen head space were both less than 1% and the precision of fill was calculated to be 0.26%. Characterization results of the freeze-dried product are summarized in Table 1.

Homogeneity of fill was confirmed at NIBSC by measurement of diphtheria antitoxin potency using the Vero cell TNT with a toxin dose level of 4xMCD. This is the most sensitive toxin dose level and allows for small differences in potency to be observed. The
assay was performed as shown in Appendix 2 but modified to use 1 starting dilution only for each test sample and toxin diluted to a stock concentration of $2.5 \times 10^5$ Lf/ml (MCD of NIBSC toxin = $6.25 \times 10^6$ Lf/ml). EC50 values were used to calculate relative activity (calculated from 4-parameter logistic dose response curve of dose vs log response). Two ampoules from the start, two from the middle and two from the end of the production run were tested. The potency estimates calculated relative to the 1\textsuperscript{st} IS 19/236 were comparable for all samples tested (GCV 3.8\% for the potency estimates across the 6 ampoules). Additional \textit{in vitro} TNT assays performed at 4xMCD showed there to be negligible loss of biological activity on freeze-drying with the pre-fill material (after dilution but prior to freeze-drying) and finished product having comparable potency estimates and suggesting an approximate recovery of 97\% of biological activity after freeze-drying.

\textbf{Collaborative study design and methods}

\textbf{Study design}

The collaborative study was organised by NIBSC (code CS667) with the aim of calibrating the candidate standard 18/180 in terms of the 1\textsuperscript{st} IS for Diphtheria Antitoxin Equine (code 19/236). Ampoules of 18/180 and 19/236 together with instructions for use were provided to all participants by NIBSC. An estimated potency for 18/180 of 50 IU/ampoule, based on preliminary post-fill potency data obtained at NIBSC, was provided to all participants to assist with preparation of dilutions. Diptheria toxin was not provided for the study and participants were asked to provide details of the toxin preparation used. They were also recommended to titrate the diptheria toxin against the reference antitoxin (DI, 19/236) in order to determine the test dose of toxin for use in the assay (if not done previously).

Participants were asked to perform at least two assays for the \textit{in vivo} method and at least three assays for the \textit{in vitro} method. Example protocols were provided and are shown in Appendix 1 (\textit{in vivo} assay) and Appendix 2 (Vero cell assay) however participants were encouraged to follow their usual procedure and to return all relevant information on assay methods and sample preparation to NIBSC on completion. The example \textit{in vivo} protocol provided by NIBSC is based on the European Pharmacopoeia monograph for diptheria antitoxin [1] and is an intradermal challenge test in guinea pigs. The \textit{in vitro} TNT performed by NIBSC is a Vero cell assay based on the method originally described by Miyamura et. al. [3] and is a modified version of the method described in the European Pharmacopoeia for Assay of Diphtheria Vaccine (Adsorbed) [7].

\textbf{Method details}

A summary of the participating laboratories with details of the methods performed and toxin dose levels used is shown in Tables 2 and 3. Six laboratories performed intradermal challenge assay as per the protocol provided (1, 2, 3, 5, 6 and 10) at a toxin dose level of Lr/100. Guinea pigs (2 per sample) were immunised with a series of six toxin/antitoxin mixtures (0.2 ml/dose, 3 per flank) and observed for 48 h for signs of erythema. Laboratory 7 performed a subcutaneous challenge test in guinea pigs at a toxin dose level of L+/1. For this assay, guinea pigs were injected with 4 ml of one of a series of 5 toxin/antitoxin mixtures (3 guinea pigs per dilution) and then observed for 120 h. Laboratories 8 and 9 performed a similar
subcutaneous challenge test using 4 guinea pigs per dilution and an observation period of 5 days.

For Vero cell TNT assays, three laboratories (2, 6, 10) performed the assay as per the protocol provided with a toxin dose level of Lcd/100 (which can be referred to as a “medium” toxin dose level). Lab 14 also performed the Vero cell assay at the Lcd/100 toxin dose level but following an in-house protocol. Laboratories (8, 12, 15 and 16) also followed an in-house protocol where the toxin dose level can be described as “low or very low” toxin (Table 3).

In all Vero cell assays, samples were titrated at least in duplicate. Titration of reference and test samples was performed using serial two-fold dilutions in laboratories 2, 6, 8, 10, 15 and 16. Laboratory 14 performed a serial 2-fold titration of the reference and a 1.5-fold titration of the test sample. Laboratory 12 prepared 6 set dilutions of the reference (1:12, 1:16, 1:20, 1:24, 1:28, 1:32) to cover a narrow range (0.03 IU – 0.003 IU) and 10 set dilutions of 18/180 (1:148 – 1:220). To obtain a more precise potency estimate, the method provided by NIBSC and that performed by laboratory 8 included 3 different starting dilutions of the test sample. The method performed by Laboratory 14 included 2 different starting dilutions. The incubation period of the toxin/antitoxin mixtures with the Vero cells ranged from 2 days to 6 days. In most laboratories end points were determined after incubation of cells with a cell viability indicator. Laboratories 7, 8 and 11 determined the cytotoxicity end points by microscopic examination.

**Stability studies**

To determine the stability of the candidate standard an accelerated degradation study and stability of the material after reconstitution was initiated at NIBSC. Stability samples were assessed in the Vero cell TNT using a toxin dose level of 4xMCD.

For the accelerated degradation study, representative samples (ampoules) were stored at +4, +20, +37, +45 and +56°C in addition to the recommended storage temperature of -20°C. Samples were put down on 30 Oct 2019 and representative samples were removed for testing after approximately 8 months. Stability was assessed by measurement of diphtheria antitoxin potency at NIBSC using the Vero cell TNT and potency was expressed relative to the same material stored at the recommended temperature of -20°C. Stability of the reconstituted material was also assessed by comparing IU values obtained for ampoules reconstituted and stored at +4°C for up to 1 year with values for ampoules reconstituted on the day of the assay.

**Reporting of data and statistical analysis**

Results from *in vivo* assays (where an intradermal challenge method was used) were reported as negative (-), positive (+) or equivocal (+/-) for each dilution injected into the shaved flank of the guinea pig. Some laboratories reported results as double (++) triple (+++) or quadruple positive (++++) to distinguish the size of the erythema. Assay end points (for the reference and test antitoxin) were taken as the dilution containing the lowest amount of antitoxin at which the first positive erythema occurred in all animals injected with the same sample. In laboratory 7 and 9, the scores (number of animals surviving at each dose) were used to
calculate ED50 values for the reference and test antitoxin, which were subsequently used to calculate relative potency values for 18/180.

For Vero cell assays, laboratories returned plate layouts showing the dilutions of reference and test antitoxin samples and the viability of cells at each dilution. Endpoints were taken as the last dilution of reference or test antitoxin able to protect Vero cells against the toxic effects of diphtheria toxin (laboratories 2, 6, 8, 10, 12, 14 and 16). Laboratory 15 reported endpoints as ED50 values.

For the purposes of calibrating the replacement IS all assays were included if an end point was obtained for both the reference and test antitoxin sample, thus allowing a relative potency calculation to be made. No further assay validity requirements applied to the end point observed for the reference sample.

**Results**

**Calibration of 18/180**

The results of the collaborative study to calibrate 18/180 are summarised in Table 4 (in vivo assays), Table 5 (in vitro Vero cell assays) and Figures 1-2. Potency is expressed relative to the 1st WHO IS for Diphtheria Antitoxin Equine (19/236) and each ampoule of the candidate standard 18/180 was reconstituted in 1 ml – therefore results expressed as IU/ml are equivalent to IU/ampoule. For in vivo potency assays the range of potency estimates obtained was 40 – 81 IU/ml and for the Vero cell assay, the range of potency estimates obtained was 50 – 84 IU/ml. The geometric mean of all laboratory means for the potency of 18/180 is 53.5 (48.9 – 59.0) IU/ml for in vivo assays (GCV 14.7%, n = 10) and 60.8 (52.9 – 70.2) IU/ml for Vero cell assays (GCV 18.3%, n = 8). This data is summarised in Table 6.

To compare results obtained in the in vivo and in vitro assays, an unpaired two-tailed t-test on the laboratory mean log10 values was used which showed no significant difference (p=0.106). If results from all assays are combined, the geometric mean of all laboratory means for the potency of 18/180 is 56.6 (52.7 – 61.9) IU/ml (GCV 17.5%, n = 18).

There was no clear relationship between the toxin dose level used and the potency estimates obtained for 18/180 (Figure 3).

One laboratory returned data obtained from an immunoassay (as opposed to a toxin neutralisation test) and reported a result of 59.5 IU/ml (n = 6 assays) which compares well to the results obtained in the toxin neutralisation tests (not shown).

One participant included the 1st WHO International Standard for Diphtheria Antitoxin Human (10/262) in each of the three Vero cell assays they performed and confirmed the assigned of 2 IU/ml for this standard.

**Stability of 18/180**

To obtain an indication of the long term stability of the candidate standard, the potency of 18/180 was determined by Vero cell TNT after storage for 8 months at elevated temperatures.
The results obtained are shown in Table 7 and potency is expressed relative to an ampoule stored at the normal storage temperature of -20°C. The 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 18/180 will be extremely stable with no loss of activity predicted when the material is stored as recommended at -20°C in the dark, and only a predicted loss of 0.002% per year if stored at a slightly higher temperature of +2°C to +8°C. These results also support shipment of the freeze-dried preparation at ambient temperature.

The analysis of stability after reconstitution suggests that 18/180 does not lose potency when stored at +2°C to +8°C in the diluted form (i.e. after reconstitution of a freeze-dried ampoule with 1 ml sterile water). Samples were tested over the course of 12 months and comparable results were obtained at all time points tested (Figure 4).

**Conclusions**

Ampoules coded 18/180 were tested and confirmed to be fit for purpose with respect to WHO recommendations [8] for precision of fill, residual moisture content and integrity – although precision of fill was marginally higher with a CV at 0.26% compared to the recommended maximum of 0.25% this is considered to be negligible in the context of the variability of assays for which this reference material is intended. Preliminary data from accelerated degradation studies indicates that the proposed standard will have adequate long-term stability.

The potency estimates obtained in the two different assay models used in the collaborative study were comparable and the variability within each assay model was low (and also comparable). The in vivo assay models remain the Pharmacopoeia methods in many regions although validation and implementation of the Vero cell assay for potency testing of equine antitoxin is likely to occur in future (and has already been implemented by some laboratories). Combining all data for assigning a potency value to 18/180 is therefore recommended and gives a value of 57 IU/ampoule. It is worth noting that the geometric mean potency from all in vivo assays is 54 IU/ampoule and a difference of 3 IU (5%) when compared to the value derived from both assay models can be considered to be negligible in the context of the assay variability in which this standard will be used.

It is recommended that 18/180 is established as the 2nd International Standard for Diphtheria Antitoxin Equine and assigned a potency of 57 IU/ampoule based on the data obtained in both assay models included in the collaborative study. This proposal was agreed by the participating laboratories.

**Recommendation**

Product coded 18/180 is recommended as a replacement for the 1st WHO IS for Diphtheria Antitoxin Equine (DI) and for adoption as the 2nd WHO IS. It is recommended that 18/180 is assigned a value of 57 IU/ampoule based on calibration by in vivo and in vitro TNT.
NIBSC will act as custodian of the standard which will be stored under assured temperature-controlled conditions within NIBSCs Centre for Biological Reference Materials, at the address listed in the introduction. A total of 2969 ampoules of 18/180 were filled at NIBSC. After collaborative study, in-house measurements and accelerated degradation studies, 2,800 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 15-20 years.

**Comments from participants**

Comments were returned from 8 participants who all agreed with the conclusions of the report. Other minor editorial comments have been addressed in preparation of this final version of the report.

**Acknowledgments**

We are extremely grateful to the participants in the collaborative study who provided the calibration data for the proposed replacement International Standard. We are also grateful to Dr Paul Matejtschuk, Sara Jane Holmes, Mark Harris and their respective teams within NIBSC who managed the formulation and production of the candidate standard, and the distribution of materials to study participants.
References


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

<table>
<thead>
<tr>
<th>NIBSC Code</th>
<th>18/180</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. Ampoules filled</td>
<td>2969</td>
</tr>
<tr>
<td>Appearance</td>
<td>Robust homogenous cake</td>
</tr>
<tr>
<td>Mean fill mass</td>
<td>1.007 g (CV 0.26%) (n=111)</td>
</tr>
<tr>
<td>Mean dry weight</td>
<td>0.006 g (CV 1.77%) (n=6)</td>
</tr>
<tr>
<td>Mean residual moisture</td>
<td>0.45% (CV 21.77%) (n=12)</td>
</tr>
<tr>
<td>Mean oxygen head space</td>
<td>0.30% (CV 41.65%) (n=12)</td>
</tr>
</tbody>
</table>

Table 2. Summary of participants and details of in vivo methods performed for the calibration of 18/180

<table>
<thead>
<tr>
<th>Lab code</th>
<th>Method identifier</th>
<th>Toxin dose level (Lf/dose)</th>
<th>No. of animals per sample</th>
<th>No. assays</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Intradermal challenge (NIBSC/EP)</td>
<td>Lr/100 (0.02)</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>Intradermal challenge (NIBSC/EP)</td>
<td>Lr/100 (0.008)</td>
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<td>2</td>
</tr>
<tr>
<td>3</td>
<td>Intradermal challenge (NIBSC/EP)</td>
<td>Lr/100 (0.01)</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>Intradermal challenge (NIBSC/EP)</td>
<td>Lr/100 (0.01)</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>Intradermal challenge (NIBSC/EP)</td>
<td>Lr/100 (0.008)</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>Intradermal challenge (NIBSC/EP)</td>
<td>Lr/100 (0.016)</td>
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<td>2</td>
</tr>
<tr>
<td>7</td>
<td>Subcutaneous challenge (In house)</td>
<td>L+/1 (not stated)</td>
<td>15</td>
<td>6</td>
</tr>
<tr>
<td>8</td>
<td>Subcutaneous challenge (In house)</td>
<td>L+/1 (not stated)</td>
<td>20</td>
<td>2</td>
</tr>
<tr>
<td>9</td>
<td>Subcutaneous challenge (In house)</td>
<td>L+/1 (not stated)</td>
<td>20</td>
<td>2</td>
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### Table 3. Summary of participants and details of in vitro Vero cell methods performed for the calibration of 18/180

<table>
<thead>
<tr>
<th>Lab code</th>
<th>Method identifier</th>
<th>Toxin dose level (Lf/ml)</th>
<th>No. plates per assay</th>
<th>No. assays</th>
</tr>
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<tbody>
<tr>
<td>2</td>
<td>NIBSC</td>
<td>Lcd/100 (0.01)</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>6</td>
<td>NIBSC</td>
<td>Lcd/100 (0.02)</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>8</td>
<td>In-house</td>
<td>~64 CD50 (^1) (not stated)</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>10</td>
<td>NIBSC</td>
<td>Lcd/100 (0.01)</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>12</td>
<td>In-house</td>
<td>Lcd/10000 (0.0002 mg/ml)</td>
<td>2 (one for reference, one for sample)</td>
<td>3</td>
</tr>
<tr>
<td>14</td>
<td>In-house</td>
<td>Lcd/100 (0.01)</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>15</td>
<td>In-house</td>
<td>MCD (IC95)(^2)</td>
<td>1-2</td>
<td>8</td>
</tr>
<tr>
<td>16</td>
<td>In-house</td>
<td>~Lcd/1000 (^3) (0.00125)</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>

\(^1\)toxin dose is approximately 64 x the 50% cytotoxic dose of the toxin used; \(^2\)toxin dose equates to the minimum cytotoxic dose for the toxin used where IC95 refers to the concentration of the toxin that results in 95% cytotoxic effect on Vero cells’ \(^3\)toxin dose equates to approximately Lcd/1000 but was not calculated by pre-titration of toxin against 0.001 IU of reference antitoxin

### Table 4. Results (IU/ampoule) for calibration of 18/180 (in vivo assays)

<table>
<thead>
<tr>
<th>Lab code</th>
<th>Method</th>
<th>Assay 1</th>
<th>Assay 2</th>
<th>Assay 3</th>
<th>Assay 4</th>
<th>Assay 5</th>
<th>Assay 6</th>
<th>Lab GM</th>
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<tbody>
<tr>
<td>1</td>
<td>i.d challenge</td>
<td>50</td>
<td>81</td>
<td>50</td>
<td></td>
<td></td>
<td></td>
<td>59</td>
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<td>2</td>
<td>i.d challenge</td>
<td>50</td>
<td>50</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>50</td>
</tr>
<tr>
<td>3</td>
<td>i.d challenge</td>
<td>50</td>
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### Table 5. Results (IU/ampoule) for calibration of 18/180 (Vero cell assays)

<table>
<thead>
<tr>
<th>Lab code</th>
<th>Assay 1</th>
<th>Assay 2</th>
<th>Assay 3</th>
<th>Assay 4</th>
<th>Assay 5</th>
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</table>
Table 6: Summary of calibration data for 18/180

<table>
<thead>
<tr>
<th>Method</th>
<th>Geometric Mean with 95% confidence limits (IU/ml)</th>
<th>GCV%</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>in vivo TNT</td>
<td>53.5 (48.9 – 59.0)</td>
<td>14.7</td>
<td>10</td>
</tr>
<tr>
<td>in vitro TNT (Vero cell)</td>
<td>60.8 (52.9 – 70.2)</td>
<td>18.3</td>
<td>8</td>
</tr>
<tr>
<td>Combined (all TNT)</td>
<td>56.6 (52.7 – 61.9)</td>
<td>17.5</td>
<td>18</td>
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</tbody>
</table>

Table 7: Accelerated degradation data (after 8 months storage) for 18/180. Data are the potency estimates for ampoules stored at elevated temperature expressed relative to samples of the same standard stored at the recommended storage temperature of -20°C. Potency was determined at NIBSC using the Vero cell TNT and a toxin dose of 4xMCD.

<table>
<thead>
<tr>
<th>Ampoule storage temperature</th>
<th>Activity relative to ampoule stored at -20°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plate 1</td>
</tr>
<tr>
<td>+4</td>
<td>1.09</td>
</tr>
<tr>
<td>+20</td>
<td>0.97</td>
</tr>
<tr>
<td>+37</td>
<td>0.95</td>
</tr>
<tr>
<td>+45</td>
<td>0.97</td>
</tr>
<tr>
<td>+56</td>
<td>0.89</td>
</tr>
</tbody>
</table>
Figure 1: Summary of potency estimates obtained in toxin neutralisation tests performed *in vivo* and *in vitro* (Vero cell). Individual data points are laboratory geometric mean estimates; bars show overall geometric means with 95% confidence limits.
**Figure 2:** Summary of potency estimates from individual assays. Each box represents an individual assay estimate and indicates laboratory code (top) and assay number (bottom).
**in vivo assays**

- assays performed with "medium toxin" dose level
- assays performed with "high toxin" dose level

**Vero cell assays**

- assays performed with "low/very toxin" dose level
- assays performed with "medium toxin" dose level
Figure 3. Potency estimates obtained in the *in vivo* (top figure) and Vero cell assays (bottom figure) grouped by toxin dose level. Data are potency estimates for 18/180 relative to 19/236 from individual assays with a line indicating the geometric mean for that laboratory.

![Stability of 18/180 after reconstitution](image)

**Stability of 18/180 after reconstitution**

Figure 4: Stability after reconstitution for 18/180. Data are potency estimates for ampoules reconstituted and stored at +4°C calculated relative to an ampoule reconstituted on the day of the assay. Data at each time point shows individual results from 3 replicate samples tested on separate plates. Potency was determined by Vero cell TNT using a toxin dose of 4xMCD.
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Centers for Disease Control and Prevention
1600 Clifton Road, MS/H23-8
Atlanta, GA 30329-4027
Appendix 1: Example protocol for in vivo assay (intradermal challenge in guinea pigs at Lr/100)

This SOP describes an assay used to determine the potency of diphtheria antitoxin by in vivo titration in guinea pigs (using intradermal challenge). The method described here is based on the European Pharmacopoeia Monograph 0086. This SOP is divided into two parts:

Part 1: titration of diphtheria toxin against the reference antitoxin (19/236) to determine the Lr/100 test dose

Part 2: titration of diphtheria antitoxin to determine antitoxin potency

The following materials and reagents are required for this assay:

Guinea pigs weighing approximately 350 – 450 g (2 per group)
Sterile saline*
Diphtheria toxin (in-house)
Reference diphtheria antitoxin (19/236) – provided by NIBSC
Test diphtheria antitoxin (18/180) – provided by NIBSC (for part 2 only)

*At NIBSC, sterile saline for this assay consists of (in 1 L):
  Sodium Chloride 8.0 g
  Potassium chloride 0.4 g
  Glucose 1.0 g

Other buffers are likely to be suitable.

Part 1. Determination of the test dose (Lr/100) of diphtheria toxin

1. Two guinea pigs with shaved flanks are required for this part of the test
2. Note that for both parts of the test, preparation of stock solutions of diphtheria antitoxin and diphtheria toxin require a minimum volume of 6 ml (prepare at least 10 ml to provide sufficient volume).
3. Prepare a solution of the reference antitoxin to contain 0.1 IU/ml.
4. Prepare a solution of the diphtheria toxin at 0.2 Lf/ml**
5. Prepare a series of 6 tubes containing mixtures of the antitoxin and toxin solutions such that each tube contains 1.0 ml of the reference antitoxin (0.1 IU/ml), one of a graded series of volumes of the diphtheria toxin solution and sufficient buffer to bring the total volume to 2.0 ml (see example in Table 1).
6. To prepare the 6 dilutions, add buffer first, followed by diphtheria toxin and finally add the fixed volume of diphtheria antitoxin.
7. Allow the mixtures to stand at room temperature (protected from light) for 30-45 min.
8. On each guinea pig, inject 0.2 ml of each of the 6 mixtures into the shaved flank. The injections should be suitably spaced so that 3 are injected on each side (6 in total).
9. After 48 h, record the incidence of specific erythema at the site of injection for all 6 sites. Any weal’s (areas of inflammation) larger than 5 mm are recorded as positive (+). Sites free from reaction are recorded as negative (-).
10. The test dose Lr/100 of diphtheria toxin is the quantity in 0.2 ml of the mixture made with the smallest amount of toxin that causes a specific erythema at the site of injection.

11. In the example shown in Table 1, the smallest amount of toxin causing an erythema is in dilution 4. This dilution contains 0.01 Lf of diphtheria toxin in the 0.2 ml injection dose. **Therefore the test dose Lr/100 in this example is 0.01 Lf.**

**Note:** the concentration of toxin shown here is an example and may need to be changed depending on the toxin preparation being used. As a guide, 1 IU of antitoxin should neutralise *approximately* 1 Lf of diphtheria toxin.

### Table 1. Dilutions for Part 1 (determine test dose Lr/100 of diphtheria toxin)

<table>
<thead>
<tr>
<th>Volume reference antitoxin (0.1 IU/ml)</th>
<th>Dilution 1</th>
<th>Dilution 2</th>
<th>Dilution 3</th>
<th>Dilution 4</th>
<th>Dilution 5</th>
<th>Dilution 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Volume Diphtheria toxin</td>
<td>0.2</td>
<td>0.3</td>
<td>0.4</td>
<td>0.5</td>
<td>0.6</td>
<td>0.7</td>
</tr>
<tr>
<td>Volume Buffer</td>
<td>0.8</td>
<td>0.7</td>
<td>0.6</td>
<td>0.5</td>
<td>0.4</td>
<td>0.3</td>
</tr>
<tr>
<td>Total volume</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Diphtheria toxin (Lf/0.2 ml dose)¹</td>
<td>0.004</td>
<td>0.006</td>
<td>0.008</td>
<td>0.01</td>
<td>0.012</td>
<td>0.014</td>
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<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

¹assuming a stock concentration of 0.2 Lf/ml

### Part 2. Determination of the potency of diphtheria antitoxin

1. Two guinea pigs with shaved flanks are required for each antitoxin sample for this part of the test (4 in total)
2. Prepare a solution of the reference antitoxin to contain 0.125 IU/ml.
3. Prepare a solution of diphtheria toxin to contain 12.5 x test dose /ml (in this example = 12.5 x 0.01 = 0.125 Lf/ml)
4. For the reference antitoxin, prepare a series of 6 tubes containing mixtures of the antitoxin and toxin such that each tube contains 0.8 ml of the diphtheria toxin solution, one of a graded series of volumes of antitoxin and sufficient buffer to bring the total volume to 2.0 ml (see example in Table 2).
5. To prepare the 6 dilutions, add buffer first followed by diphtheria antitoxin and finally add the fixed volume of diphtheria toxin.
6. Note that the series of dilutions should be centred on the mixture containing 0.01 IU of reference antitoxin in the 0.2 ml dose (dilution 3 in the example in Table 2).
7. Prepare a dilution of the test antitoxin sample to give an estimated concentration of 0.125 IU/ml (i.e. the same as the reference antitoxin).
8. Prepare a series of 6 tubes containing mixtures of the test antitoxin, diphtheria toxin and buffer exactly as described for the reference antitoxin (see Table 3 for an example) - add buffer first followed by diphtheria antitoxin and finally add the fixed volume of diphtheria toxin.

9. Allow the mixtures to stand at room temperature (protected from light) for 30-45 min.

10. Using 2 guinea pigs for each series of dilutions, inject 0.2 ml of each of the 6 mixtures into the shaved flank. The injections should be suitably spaced so that 3 are injected on each side (6 in total).

11. After 48 h, record the incidence of specific erythema at the site of injection for all 6 sites. Any weal’s (areas of inflammation) larger than 5 mm are recorded as positive (+). Sites free from reaction are recorded as negative (-).

12. For the test antitoxin, the dilution containing the largest volume of antitoxin that fails to protect the guinea pigs from erythema contains 0.1 IU in the total volume of 2 ml. This quantity is used to calculate the antitoxin potency as follows:

13. In the example in Table 3, the largest volume of antitoxin failing to protect guinea pigs is in dilution 3. This dilution contains 0.8 ml of the antitoxin dilution. Therefore, 0.8 ml contains 0.1 IU and 1 ml contains 0.125 IU antitoxin. This figure is multiplied by the dilution factor of the original stock solution – in this example = 1/400 so 0.125 x 400 = 50 IU/ml.
Table 2. Dilutions for Part 2 – preparation of reference antitoxin samples (all volumes in ml)

<table>
<thead>
<tr>
<th></th>
<th>Dilution 1</th>
<th>Dilution 2</th>
<th>Dilution 3</th>
<th>Dilution 4</th>
<th>Dilution 5</th>
<th>Dilution 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume reference antitoxin (0.125 IU/ml)</td>
<td>1.2</td>
<td>1.0</td>
<td>0.8</td>
<td>0.62</td>
<td>0.5</td>
<td>0.4</td>
</tr>
<tr>
<td>Volume Diphtheria toxin (12.5 x test dose /ml)</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
</tr>
<tr>
<td>Volume Buffer</td>
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<td>0.4</td>
<td>0.58</td>
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<td>0.8</td>
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<td>Total volume</td>
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<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
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<td>Antitoxin IU/0.2 ml dose</td>
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<td>0.0125</td>
<td>0.010</td>
<td>0.00775</td>
<td>0.00625</td>
<td>0.005</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 3. Dilutions for Part 2 – preparation of test antitoxin samples (all volumes in ml)

<table>
<thead>
<tr>
<th></th>
<th>Dilution 1</th>
<th>Dilution 2</th>
<th>Dilution 3</th>
<th>Dilution 4</th>
<th>Dilution 5</th>
<th>Dilution 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume test antitoxin (example diluted 1/16)</td>
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<td>1.0</td>
<td>0.8</td>
<td>0.62</td>
<td>0.5</td>
<td>0.4</td>
</tr>
<tr>
<td>Volume Diphtheria toxin (12.5 x test dose /ml)</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
</tr>
<tr>
<td>Volume Buffer</td>
<td>0.0</td>
<td>0.2</td>
<td>0.4</td>
<td>0.58</td>
<td>0.7</td>
<td>0.8</td>
</tr>
<tr>
<td>Total volume</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Example Erythema</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Appendix 2: Example protocol for in vitro Vero cell assay (at Lcd/100)

This SOP describes an assay used to determine the potency of diphtheria antitoxin by in vitro titration using Vero cells. This SOP is divided into two parts:

**Part 1:** titration of diphtheria toxin against the reference antitoxin (19/236) to determine the Lcd/100 test dose (Lcd/100 refers to the smallest amount of toxin neutralised by 0.01 IU of reference antitoxin) – this must be done before Part 2 is started.

**Part 2:** titration of diphtheria antitoxin to determine antitoxin potency

The following materials and reagents are required for this assay:

- VERO
- Complete cell culture medium (abbreviated to CCM)*
- 96 well flat bottom tissue culture microplate with lid
- Diphtheria toxin (in-house)
- Reference diphtheria antitoxin (19/236) – provided by NIBSC
- Test diphtheria antitoxin (18/180) – provided by NIBSC (for part 2 only)
- Phosphate Buffered Saline pH 7.4
- Thiazolyl Blue Tetrazolium Bromide (MTT, Sigma M2128)
- MTT extraction buffer (10% w/v SDS in 50% v/v dimethylformamide, pH 4.7)
- Multichannel pipettes

*At NIBSC CCM is prepared using minimum essential medium (MEM, Sigma M2279) supplemented with 5% fetal bovine serum, 1x antibiotic-antimycotic solution, 2mM L-Glutamine, 0.1% D-Glucose and 0.015M HEPES).

**Part 1. Determination of the test dose (Lcd/100) of diphtheria toxin**

1. Prepare a dilution of the reference antitoxin (19/236) in CCM to give a stock solution of 0.01 IU/ml (prepare at least 10 ml per plate).
2. Prepare a dilution of diphtheria toxin in CCM to give a stock solution of 0.16 Lf/ml (approximately 1 ml per plate is required).
3. Prepare a flat bottom 96 well tissue culture microplate by marking the columns for samples and controls as shown in the example Figure 1.
4. Add 150 µl of CCM to all blank control wells; add 100 µl CCM to all cell control wells.
5. Add 50 µl CCM to all sample wells – EXCEPT for the top row (A) which remains empty.
6. Add 100 µl of diluted diphtheria toxin stock to the appropriate wells in row A. Titrate down the plate (serial two-fold dilutions of 50 µl volumes) and discard 50 µl from row H.
7. Add 50 µl of diluted diphtheria antitoxin stock (0.01 IU/ml) to all sample wells. These wells now contain a total volume of 100 µl. Shake the plate gently by hand, cover with a lid and leave at room temperature for 1h to allow neutralisation to occur.
8. Meanwhile, prepare a suspension of Vero cells in CCM at a density of approximately 4 x 10^5 cells per ml.
9. Add 50 µl of the Vero cell suspension to all sample wells and the cell control well. These wells now contain a total volume of 150 µl.
10. Cover with a lid, shake the plates gently by hand and incubate in a 5% CO2 incubator at +37 °C for 6 days. A plate sealer (pressure film) may be added to seal the plate before incubation if required.
11. After 6 days, perform the MTT viability assay to determine assay end points.
12. Prepare a solution of MTT at 5 mg/ml in PBS – pass though a 0.2 µM filter.
13. Add 10 µl of this MTT solution to all wells in the plate (including controls); return the plate to the incubator for approximately 4 h.
14. Remove the supernatant from all wells using a multichannel pipette set to >150 µl.
15. Add 100 µl of MTT extraction buffer to all wells and return the plate to the incubator overnight to allow complete extraction of the formazan product to occur.
16. Read the absorbance at ~570 nm.

**Figure 1.** Example of the plate layout for determining the Lcd/100 test dose of diphtheria toxin.

<table>
<thead>
<tr>
<th>Plate 1</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Spare</td>
<td>Spare</td>
<td>Spare</td>
<td>Toxin 0.16 Lf/ml</td>
<td>Toxin 0.16 Lf/ml</td>
<td>Spare</td>
<td>Spare</td>
<td>Spare</td>
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**Note:** in this example the toxin is titrated in duplicate. More replicates should be included where possible. In addition, duplicate plates can be prepared if necessary.

**Calculation of results and determination of assay end points**

An example of the results obtained is shown in Figure 2. The average (or geometric mean) OD570 for the cell control (column 11) is calculated and divided by 2 to give a cut-off value which defines the assay end point.

In this example, the OD geometric mean for the cell control = 1.34; therefore the 50% control OD is 1.342 / 2 = **0.671**.

The Lcd/100 is defined as the lowest concentration of diphtheria toxin that causes cytotoxicity in the presence of 0.01 IU/ml of reference diphtheria antitoxin. In the example shown in Figure 2, cytotoxicity is defined as an OD <0.671 and dilutions 1-5 are all toxic. The lowest concentration of toxin is in dilution 5 (0.01 Lf/ml) so the Lcd/100 = **0.01 Lf/ml**.
Figure 2. Example of results obtained in the Vero cell assay (determination of Lcd/100)

<table>
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<tr>
<th>Plate 1</th>
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</table>

Part 2. Determination of the potency of diphtheria antitoxin

Note, in order to obtain a more precise potency estimate for the candidate diphtheria antitoxin, the sample can be tested at three different starting dilutions; the dilution to give an estimated concentration equivalent to that of the reference antitoxin 0.16 IU/ml (1/312.5), and then at 2 extra dilutions equally spaced (on a log scale) between 1/312.5 and 1/625 (the next dilution in the 2-fold titration series).

Dilution 1: 1/312.5

Example: 1/10 followed by 1/31.25
1/10: 100 µl 18/180 + 900 µl CCM
1/312.5: 32 µl of 1/10 dilution + 968 µl CCM

Dilution 2: 1/393.7

Example: 1/10 (see above) followed by 1/39.37
1/393.7: 25.4 µl of 1/10 dilution + 974.6 µl CCM

Dilution 3: 1/495

Example: 1/10 (see above) followed by 1/49.5
1/495: 20.2 µl of 1/10 dilution + 979.8 µl CCM

1. Prepare a dilution of the reference antitoxin (19/236) in CCM to give a stock solution of 0.16 IU/ml (prepare at least 1 ml per plate).
2. Prepare dilutions of the test antitoxin sample in CCM to give 3 different stock solutions; one with an estimated concentration equivalent to that of the reference antitoxin (so for 18/180 which has an estimated potency of 50 IU/ml, this would be 1/312.5) and then two others equally spaced before the next dilution in the doubling dilution series (1/393.7 and 1/495) to obtain a more precise estimate (see above for examples).
3. Prepare a dilution of diphtheria toxin in CCM to contain the Lcd/100 test dose per ml (using the example from Part 1, the stock solution will contain 0.01 Lf/ml) – prepare at least 10 ml per plate.
4. Prepare the 96 well plate (see example layout shown in Figure 3). The reference antitoxin and three different dilutions of the test antitoxin should be tested in duplicate on each plate. It is recommended to include three replicate plates where possible.
5. Add 150 µl CCM to all blank control wells; add 100 µl CCM to the cell control wells (column 11 in figure 3). Add 50 µl CCM to the toxin control wells (column 2 in Figure 3).
6. Add 50 µl CCM to all sample/reference antitoxin wells (columns 3-10 in Figure 3) – EXCEPT for the top row (A) which remains empty.
7. Add 100 µl of diluted reference antitoxin or test antitoxin to the appropriate wells in row A. Titrate down the plate (serial two-fold dilutions of 50 µl volumes) and discard 50 µl from row H.
8. Add 50 µl of diluted diphtheria toxin stock to all wells containing reference or test antitoxin (columns 3-10 in Figure 3). Add 50 µl of diluted diphtheria toxin to the toxin control wells (column 2 in Figure 3). Shake the plate gently by hand, cover with a lid and leave at room temperature for 1h to allow neutralisation to occur.
9. Meanwhile, prepare a suspension of Vero cells in CCM at a density of approximately 4 x 10^5 cells per ml.
10. Add 50 µl of the Vero cell suspension to all wells except for blanks (i.e. columns 2-11 in figure 3). All wells now contain a total volume of 150 µl.
11. Cover with a lid, shake the plates gently by hand and incubate in a 5% CO₂ incubator at +37 °C for 6 days. A plate sealer (pressure film) may be added to seal the plate before incubation if required.
12. After 6 days, perform the MTT viability assay to determine assay end points as described in part 1.

**Figure 3.** Example of plate layout to determine the potency of diphtheria antitoxin

<table>
<thead>
<tr>
<th>Plate 1</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
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<tbody>
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<td></td>
<td>Ref 19/236 0.16</td>
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<td>Test ATx Dil.2</td>
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<td>Ref 19/236 0.16</td>
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<td></td>
</tr>
<tr>
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</tr>
<tr>
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</tr>
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</table>

**Note:** In this example, dilutions for the test antitoxin are dilutions in the plate, not total dilution
Calculation of results and determination of assay end points
An example of the results obtained is shown in Figure 4. For the Vero cell analysis to be valid all the cell control wells must show positive cell growth and all of the toxin control wells must show toxicity and cell death.

The average (or geometric mean) OD570 for the cell control (column 11) is calculated and divided by 2 to give a cut-off value which defines the assay end point.

In this example, the OD geometric mean for the cell control = 1.346; therefore the 50% control OD is 1.346 / 2 = 0.673.

The end point of the assay is the lowest dilution of antitoxin at which cells are protected from the toxic effects of the diphtheria toxin (i.e. OD > 0.673). In the example shown in Figures 3 and 4, the end point for the reference antitoxin is at 0.02 IU/ml.

The end point for the test antitoxin is at 1/8 on the plate for all 3 stock solutions. Potency for each dilution is calculated using the following formula, and the geometric mean of all three results is taken to obtain the final estimated potency:

End point dilution in plate x Dilution of stock x End point for reference antitoxin IU/ml
- Dilution 1: 8 x 312.5 x 0.02 = 50 IU/ml
- Dilution 2: 8 x 393.7 x 0.02 = 63 IU/ml
- Dilution 2: 8 x 495 x 0.02 = 79 IU/ml
- Final geometric potency = 63 IU/ml

Figure 4. Example of results obtained in the Vero cell assay (potency of diphtheria antitoxin)

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Appendix 3: Instructions for Use (draft)

9. REFERENCES
[1] Reference to WHO BS report to be added

10. ACKNOWLEDGEMENTS
NIBSC is grateful to the participants in the collaborative study who provided data to support the calibration and establishment of this reference preparation [1]

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

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

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<tr>
<td>Not established, avoid ingestion</td>
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<tr>
<td>Effects of skin absorption:</td>
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<tr>
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</table>

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.
15. LIABILITY AND LOSS
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   - Country of origin for customs purposes*: United Kingdom
   - Defined as the country where the goods have been produced and/or sufficiently processed to be classified as originating from the country of supply, for example a change of state such as freeze-drying.
   - Not weight: Mean dry weight 6.1 mg; total weight per ampoule 3.88g
   - Toxicity Statement: Non-toxic
   - Veterinary certificate or other statement if applicable. Attached: No

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