EXPERT COMMITTEE ON BIOLOGICAL STANDARDIZATION
Geneva, 24 to 28 October 2022

Collaborative study: Calibration of 1st WHO Reference Reagent for Tetanus Antitoxin Equine for use in Flocculation Test

Shalini Rajagopal, Robert Tierney, Peter Rigsby, Paul Stickings

National Institute for Biological Standards and Control, Medicines and Healthcare products Regulatory Agency, Blanche Lane, South Mimms, Potters Bars, UK.

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 23 September 2022 and should be addressed to the attention: 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.

© World Health Organization 2022

All rights reserved.

This draft is intended for a restricted audience only, i.e. the individuals and organizations having received this draft. The draft may not be reviewed, abstracted, quoted, reproduced, transmitted, distributed, translated or adapted, in part or in whole, in any form or by any means outside these individuals and organizations (including the organizations' concerned staff and member organizations) without the permission of the World Health Organization. The draft should not be displayed on any website.

Please send any request for permission to:

Dr Ivana Knezevic, Technical Standards and Specifications, Department of Health Products Policy and Standards, World Health Organization, CH-1211 Geneva 27, Switzerland. Email: knezevici@who.int.

The designations employed and the presentation of the material in this draft do not imply the expression of any opinion whatsoever on the part of the World Health Organization concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. Dotted lines on maps represent approximate border lines for which there may not yet be full agreement.

The mention of specific companies or of certain manufacturers’ products does not imply that they are endorsed or recommended by the World Health Organization in preference to others of a similar nature that are not mentioned. Errors and omissions excepted, the names of proprietary products are distinguished by initial capital letters.
Summary

We report here the results of a study for the characterization of a preparation of tetanus antitoxin equine (coded 21/230) and its fitness for purpose as a standardized reagent for the flocculation (Lf) test. The candidate reference reagent was characterised using the Ramon flocculation method, standardized using the 3rd International Standard (IS) for Tetanus Toxoid for use in Flocculation Test (16/302). The preparation coded 21/230 was shown to be suitable for use as a reference reagent in the Lf test, with an estimated 912 Lf-eq/ampoule with 95% CI of 878-948, and an average Kf time of 15 minutes, based on results from 9 laboratories in 8 countries. There was good agreement in the results obtained (intra-laboratory GCV ranging from 0% to 6.9%, interlaboratory GCV 5.2%).

Data from accelerated thermal degradation studies showed no temperature dependent loss in flocculation activity after storage for 3 months at elevated temperatures, indicating that the reference reagent is likely to have good long-term stability. Further studies at later timepoints will be performed post establishment.

Based on the results of this study, and with the agreement of the participants, it is proposed that 21/230 be established as the 1st WHO Reference Reagent for Tetanus Antitoxin Equine for use in Flocculation Test. The reference reagent should be pre-calibrated against the WHO IS for Tetanus Toxoid for use in Flocculation Test 16/302 within each laboratory and no units are assigned to this reference reagent.
Introduction

Tetanus is a potent neurotoxin produced by the bacterium, *Clostridium tetani*. Tetanus toxoid is the major component of tetanus vaccines, which are usually administered in combination with diphtheria and pertussis as DTP vaccines. Tetanus toxoid is also used as a carrier protein in several polysaccharide vaccines, including *Hemophilus influenzae* and *Neisseria meningitidis*. Tetanus toxin is isolated from toxigenic strains of *C.tetani* cultures and inactivated with formaldehyde to produce tetanus toxoid, then the toxoid is purified and sterilized to produce the toxoid antigen [1].

The content or concentration of toxoid in a sample can be expressed as a flocculation value (Lf), also known as the limit of flocculation, using the Ramon flocculation assay. Toxoid purity is measured in Lf units per milligram of protein nitrogen (PN) and tetanus toxoids used in the production of vaccines for human use must be shown to meet minimum requirements for purity. The World Health Organisation (WHO) minimum requirements for antigenic purity of bulk tetanus toxoid components is not less than 1000 Lf/mg of PN for use in the production of vaccines for human use [1]. This minimum requirement of 1000 Lf/mg PN is an essential quality criterion for the toxoid. Measurement of antigen content in Lf also indicates the consistency of toxoid production.

Flocculation assay is an *in vitro* immunology binding assay used to determine the Lf of a toxoid (or toxin). It is routinely used for quantifying diphtheria and tetanus toxoid content during vaccine production, and requires a hyperimmune equine diphtheria or tetanus antitoxin as a critical reagent. In the Ramon flocculation assay, antitoxin is added in increasing concentrations to a series of tubes containing a fixed amount of antigen (toxoid). The flocculation reaction occurs at the point of equivalence as a result of the formation of antigen-antibody complexes, and visible floccules appear, in one (or more) tube first. The first tube in which flocculation occurs is used to calculate the Lf value of the toxoid sample, taking into account any initial dilutions [2]. The time taken for the first tube to flocculation is known as Kf and it is a useful indicator of the quality of the antigen and antitoxin used. Alternative flocculation methods include the Dean-Webb test where the antigen concentration is varied and the antitoxin concentration is fixed, or the Levine-Wyman test where both the antigen and antitoxin concentrations are varied at the same time [3].

Historically, the flocculation unit was a relative unit, defined as the amount of toxoid (or toxin) equivalent to one International Unit (IU) of (diphtheria or tetanus) antitoxin in the Ramon flocculation test [3]. When the second International Standard for Tetanus Antitoxin (TE) was endorsed by the WHO Expert Committee on Biological Standardization, the committee also discussed a suggestion to define the Lf unit, not by a relationship to the antitoxin unit, but directly by means of a standard toxoid preparation [4]. Following this, the 1st International Reference Reagent of Tetanus Toxoid for Flocculation Test (TEFT) was established in 1988, calibrated in Lf/ampoule against TE, which had the valid Lf definition at the time [5,6]. TEFT was replaced by the 2nd IS for Tetanus Toxoid for use in Flocculation Test (04/150) in 2007, and this was subsequently replaced by the 3rd IS for Tetanus Toxoid for use in Flocculation Test (16/302) in 2019 [6,7]. The main advantage of shifting the calibration approach to use of an
International Standard toxoid preparation was that any suitable antitoxin could then be used as a reagent in the flocculation test.

Laboratories conducting flocculation assays for tetanus toxoid therefore now pre-calibrate a suitable antitoxin in Lf-eq units against the WHO IS for toxoid. Currently, 16/302 with an assigned unitage of 970 Lf/ampoule is used for this pre-calibration step [7]. The pre-calibrated antitoxin is then used to calculate Lf values of unknown toxoid or toxin samples. In this way, Lf concentrations of tetanus toxoids are fully traceable to the IS (16/302).

Tetanus antitoxin equine for flocculation assay is produced from hyperimmune horse serum and therefore has a very high concentration of antitoxin, which is key for its designated purpose as a flocculation assay reagent [8]. A lyophilized preparation of hyperimmune equine antitoxin (product 66/021) was previously available from NIBSC as a non-WHO reference reagent (RR). This reagent was widely used and stocks are now completely depleted. This report summarises the development of a new lyophilized antitoxin reagent and its evaluation in a small international collaborative study.

**Bulk material and processing**

Five litres of tetanus antitoxin equine were obtained from Wirtschaftsgenossenschaft deutscher Tierärzte (WDT, Germany), and processed for filling and freeze-drying on 23 September 2021. The material was filled using a Bausch & Strobel filling machine (AFV5090) and freeze-dried using Serail CS100 freeze dryer (Le Coudray St Garmer, France) and a standard plasma cycle (FD0064, 4-day cycle). The suitability of the material, both pre-lyophilization and post-filling/lyophilization, was confirmed in flocculation assays at NIBSC.

**Characterization of freeze-dried candidate standards**

After filling and freeze-drying, the candidate antitoxin (coded 21/230) was examined for appearance, residual moisture content and oxygen head space. Additionally, tetanus neutralizing activity and flocculating activity were also determined.

**Tetanus Neutralisation Assay**

The candidate reference reagent 21/230 was tested for tetanus antitoxin potency at NIBSC using the mouse tetanus toxin neutralisation assay (TNT), with the potency estimated in International Units (IU) relative to Tetanus Antitoxin equine (TE) (previously 2nd International Standard (IS) for Tetanus Antitoxin Equine). The test is based on the European Pharmacopoeia (Ph Eur) monograph 0091 for tetanus antitoxin with the toxin dose level adjusted to Lp/200 to allow a higher assay sensitivity. The assay was performed using NIH mice (males and females, 16–20 g; Envigo UK), taking the onset of localized tetanus paralysis in the injected hind limb as the end-point. A series of dilutions of a reference standard and 21/230 were made in gelatine-PBS (GPBS) in the presence of a fixed amount of tetanus toxin (Lot AWX4664). The mixtures were allowed to stand for 30 minutes at room temperature prior to injection (0.5 ml s.c., left thigh), with each dilution group consisting of 4 mice. The mice were observed for 96 hours for signs of tetanus paresis. The protective capacity of 21/230 was compared to the amount of the reference antitoxin standard required to give 50% protection against the paralytic effect of tetanus toxin. Protective concentration was expressed in IU. This study was performed under a Project Licence
granted by the UK Home Office (P6014F8B4) and reviewed/approved by the NIBSC Animal Welfare and Ethical Review Body.

Flocculation Assays
Flocculation assays performed at NIBSC were carried out using the Ramon flocculation method, where the antigen concentration is kept constant and different amounts of antitoxin are added to a series of tubes. For pre-calibration of 21/230, the tetanus antitoxin was reconstituted in 1.0 ml water – therefore results expressed as Lf-eq/ml are equivalent to Lf-eq/ampoule. A series of seven tubes, containing 0.50 to 0.80 IU of antitoxin and 50 Lf of the 3rd IS for Tetanus Toxoid for use in Flocculation Test (16/302), were prepared in 0.9% saline to a total volume of 2 ml. Tubes were incubated in a water bath at 50°C, and checked at regular intervals to observe when flocculation had taken place. Based on the 1st tube to flocculate (which would contain 50 Lf-eq units of antitoxin), the Lf-eq value of 21/230 was calculated taking into account the initial dilution factor.

For in-house quantification of two tetanus toxoid samples, “Toxoid A” and “Toxoid B”, the pre-calibrated candidate 21/230 or non-WHO equine antitoxin 66/021 were diluted to 50 Lf-eq/ml (both based on pre-calibration against 16/302). Using the Ramon flocculation assay, a series of seven tubes, containing 0.35 to 0.65 IU of antitoxin and ~50 Lf/ml of the tetanus toxoids, were prepared in 0.9% saline to a total volume of 2 ml. The first tube in which flocculation appeared was used to calculate the Lf value of the toxoid sample, with the order of flocculation recorded for the first, second and third tubes to flocculate. After the initial broad range assays for toxoids A and B, narrow range assays were used to determine a more precise Lf/ml estimate (with 2 Lf increases from tube to tube). The narrow range assays for each toxoid were performed three times.

Collaborative study design and methods

Study design
21/230 will not have a formal assigned unitage because it will be pre-calibrated within each laboratory against the IS for Tetanus Toxoid for use in Flocculation Test. A small international collaborative study (coded CS701) was run to demonstrate fitness for purpose of 21/230. Participants were provided with flocculation method guidelines based on established methods from the World Health Organisation (WHO) and Ph Eur, and asked to determine the Lf-eq value for 21/230 using the Ramon flocculation method.

Flocculation assays
Participants were asked to pre-calibrate the candidate antitoxin in Lf-eq against the 3rd International Standard Tetanus Toxoid for use in Flocculation Test, 16/302, using one broad range assay and three narrow range assays. Each laboratory was provided with sufficient ampoules of 16/302 and 21/230 to perform four independent assays using new ampoules for every test. To assist with preparation of a suitable dilution range, 21/230 was labelled with a nominal potency value of 1400 IU/ml, based on the result of the in vivo potency test performed at NIBSC, to aid users in preparing the dilution series of the antitoxin.
The participants were recommended to prepare a series of seven tubes containing 0.50 to 0.80 IU of antitoxin and 50 Lf of the 3rd IS for tetanus toxoid for use in flocculation test (16/302), 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 the original antitoxin sample, taking any initial dilution factor into account, and to use this Lf-eq value for subsequent assays to narrow the range for more accurate estimation of the Lf-eq for 21/230. If flocculation occurred in either the first or the last antitoxin dilution of the range, the participants were asked to repeat the assay using a different range of antitoxin dilutions.

**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 deviations from the method guidelines were reported to NIBSC.

Results from all the narrow range assays were combined as unweighted geometric means (GM) for each laboratory, and these laboratory means were used to calculate the overall unweighted geometric mean. Variability between assays and laboratories has been expressed using geometric coefficients of variation (GCV = \(10^{s-1}\)x100\% where \(s\) is the standard deviation of the \(\log_{10}\) transformed estimates).

**Stability studies**
To determine the stability of the candidate reference reagent, an accelerated degradation study was initiated at NIBSC. For this study, representative ampoules of 21/230 were stored at +4, +20, +37, +45 and +56°C in addition to the recommended storage temperature of -20°C. Data were obtained using flocculation assays after 3 months of storage at each temperature, with further data to be collected at later time points (up to 5 years).

**Results**

**Characterization of freeze dried product 21/230**
The lyophilized product was of very good appearance, with a robust and homogenous cake. The precision of fill was determined by weighing ampoules from across the production run post fill. A total of 190 ampoules were weighed and the mean fill mass was 1.001g with a coefficient of variation (CV) of 0.29\%. Ampoules were sealed under boiled off gas from high purity liquid nitrogen (99.99\%) and measurement of the mean oxygen head space after sealing was used as a measure of ampoule integrity. The mean dry weight was 0.035g with a CV of 0.37\%. 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 the WHO requirements for reference materials, with 0.10\% mean residual moisture and 0.08\% mean oxygen head space. Characterization results for 21/230 are summarised in Table 1.

21/230 had an estimated tetanus potency of 1361 IU/ampoule from the TNT assay. This indicated an approximate 91\% recovery of biological activity post freeze-drying compared to the
potency estimate obtained with the pre-filled liquid source material. From this result, the collaborative study participants were provided with a nominal potency of 1400 IU/ampoule to use as a starting point for preparing dilutions in the flocculation assay. A small reduction in Lf-eq value was also observed for 21/230 post lyophilization with 1025 Lf-eq/ml (n=3) compared with 1077 Lf-eq/ml (n=1) for the liquid bulk antitoxin.

**Collaborative study results**

Flocculation assay results were returned by 9 laboratories including NIBSC. The flocculation assay was the sole test requested for this collaborative study, as this is the proposed primary purpose for this reference reagent (although it may also be suitable for use in other bioassays). The majority of the labs chose to perform the ‘broad’ range according to the dilution range suggested in the method guidelines, followed by a ‘narrow’ range using smaller increases from tube to tube to obtain a more precise estimate of Lf-eq. The labs performed at least four independent assays, one broad range (assay 1) and three narrow range (assays 2, 3 and 4), apart from Lab 1 (three narrow range assays only).

All raw data together with assay details were returned to NIBSC (using provided data sheets). Any deviations from the method guidelines were reported to NIBSC. Results from the three narrow range assays were combined and used to calculate an unweighted GM for each laboratory, and these laboratory means were used to calculate the overall unweighted GM. Variability between the laboratories and the narrow range assays were expressed using GCVs. Table 2 summarises the results (Lf-eq/ml) obtained for 21/230 in the Ramon flocculation test from the collaborative study laboratories for individual assays and the overall GM, GCV and 95% confidence intervals. An overall geometric mean of 912 Lf-eq/ml was determined for 21/230, with 95% confidence intervals of 878-948 (GCV 5.2%), calculated from the three narrow range assays (Table 2). The GMs from across the labs ranged from 854 to 996 Lf-eq/ml, with within-laboratory GCVs for Lf-eq/ml ranging from 0% (all assays giving the same results) to 6.9%. Figure 1 shows a summary of all broad range and narrow range data from each laboratory in Lf-eq/ml. The average Kf times observed for 21/230 are also shown in Table 2 and the average Kf ranged from 10 to 19 minutes.

**Stability studies**

Flocculation assays were carried out on ampoules stored at temperatures ranging from -20 (baseline) to +56°C after three months post the definitive fill, in a single laboratory (NIBSC, Table 3). Data from this accelerated degradation study showed no change in 21/230 flocculation activity measured in Lf-eq/ml from -20 to +45°C, and a slight reduction in Lf-eq/ml activity with storage at +56°C after 3 months. Storage at temperatures from -20 to +20°C gave consistent Kf times of 14 minutes. Storage of 21/230 at temperatures above +20 and up to +56°C led to slightly increased Kf times from a baseline of 14 mins up to 23 mins. All samples reached the end point of visible flocculation and results suggested no expected issues for longer term stability. No prediction of stability could be made as no loss in activity relative to the -20°C baseline was observed at temperatures up to +45°C, however further studies will be performed at later time points (up to 5 years).
Fitness for Purpose by Flocculation Test

21/230 was used to quantify two bulk tetanus toxoid samples (A and B) from a vaccine manufacturer using the flocculation assay, with parallel quantification conducted with the current non-WHO reference reagent coded 66/021. Toxoid A had a labelled value of 5250 Lf/ml and Toxoid B had a labelled value of 2160 Lf/ml. Results shown (Table 4) are from three narrow range flocculation assays for each toxoid and antitoxin, with Kf times in minutes shown in brackets. The Lf/ml results obtained here for 21/230 and 66/021 were very similar for the two tetanus toxoids A and B. These results provide some assurance that users who switch from 66/021 to 21/230 are likely to obtain good continuity of results for Lf measurement of tetanus toxoid samples.

The time taken for the first flocculation to occur (Kf) is known to vary dramatically depending on the antitoxin used. For 21/230, the Kf times were noticeably shorter for both toxoids A and B (mean Kf times of 31 and 14 mins respectively), compared to 66/021 (mean Kf times of 67 and 29 mins respectively). This is likely due to differences in the production of the two antitoxins: 66/021 was produced from undigested serum from a single horse immunized with toxoid followed by immunization with toxin, whereas 21/230 was produced from a pool of equine serum which was subsequently F(ab)2 purified [8].

Summary and Recommendation

Ampoules coded 21/230 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 indicate that the proposed reference reagent will have suitable long-term stability.

The results from this study confirm that the tetanus antitoxin equine preparation coded 21/230 is suitable as the 1st WHO Reference Reagent for Tetanus Antitoxin Equine for use in Flocculation Test. There is no formally assigned unitage for this reference reagent but the instructions for use will contain the estimated potency in IU/ampoule to aid users in the preparation of dilutions for pre-calibration of this reference reagent against the WHO IS Tetanus Toxoid for use in Flocculation Test. As noted in the introduction, there is no requirement for an International Standard Antitoxin for flocculation testing. However, given that hyperimmune equine tetanus antitoxin is very difficult for many laboratories to obtain, the availability of a stable, well characterized WHO reference reagent will facilitate the performance of this routine test in laboratories worldwide. NIBSC will act as custodian of the reference reagent which will be stored under assured temperature-controlled conditions within the Institute’s Centre for Biological Reference Materials.

A total of 5024 ampoules of 21/230 were filled at NIBSC. After the collaborative study, in-house measurements, and accelerated degradation studies, 4832 ampoules remain available at NIBSC (-20°C). Based on current use, it can be predicted that this will be sufficient for 10-15 years.

Comments from participants

All 8 participants (not including NIBSC) were sent a draft report and asked to comment on the content and conclusions, and to confirm that their results had been reported correctly. Six
participants responded (75%) and four confirmed they agreed with the content and recommendations. Data were reported incorrectly for one participant (lab 5) for the broad range flocculation assay, and for one participant for the narrow range flocculation assays (lab 2). These data were subsequently corrected in Table 1, and the errors did not change the overall GM of 912 Lf-eq/ml from the collaborative study. Labs 2 and 5 then confirmed they agreed with the content and recommendations. Lab 4 provided feedback on the interpretation of Figure 1 and additional descriptive text was included in the report referring to Figure 1.

Acknowledgments
We are extremely grateful to all the participants who took part in the collaborative study. At NIBSC, Dr. Paul Matejtschuk and Kiran Malik (Standardisation Science) are acknowledged for their contribution to the lyophilization studies, and the staff of Standards Processing Division for production of the RR and distribution of materials for the collaborative study. We would also like to thank Sanofi Pasteur for the tetanus toxoids provided for the fitness for purpose studies.

Abbreviations Used

References


Table 1. Summary of stabilised, freeze-dried candidate reference reagent

<table>
<thead>
<tr>
<th>NIBSC Code</th>
<th>21/230</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antitoxin Manufacturer</td>
<td>WDT</td>
</tr>
<tr>
<td>No. Ampoules filled</td>
<td>5024</td>
</tr>
<tr>
<td>Appearance</td>
<td>Robust homogenous cake</td>
</tr>
<tr>
<td>Mean fill mass</td>
<td>1.001g (CV 0.29%) (n=190)</td>
</tr>
<tr>
<td>Mean dry weight</td>
<td>0.035g (CV 0.37%) (n=6)</td>
</tr>
<tr>
<td>Mean residual moisture</td>
<td>0.10% (CV 11.81%) (n=12)</td>
</tr>
<tr>
<td>Mean oxygen head space</td>
<td>0.08% (CV 41.81%) (n=12)</td>
</tr>
</tbody>
</table>

Table 2: Flocculation test results (Lf-eq/ampoule) for 21/230. BR: broad range, NR: narrow range, ND: no data. Average Lf-eq/ml results calculated from NR assays only.

<table>
<thead>
<tr>
<th>Lab</th>
<th>Assay 1 BR Lf-eq/ml Kf time</th>
<th>Assay 2 NR Lf-eq/ml Kf time</th>
<th>Assay 3 NR Lf-eq/ml Kf time</th>
<th>Assay 4 NR Lf-eq/ml Kf time</th>
<th>Average Lf-eq/ml</th>
<th>Average Kf time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ND</td>
<td>15</td>
<td>909</td>
<td>15</td>
<td>933</td>
<td>14</td>
</tr>
<tr>
<td>2</td>
<td>875</td>
<td>13</td>
<td>854</td>
<td>16</td>
<td>854</td>
<td>18</td>
</tr>
<tr>
<td>3</td>
<td>875</td>
<td>21</td>
<td>959</td>
<td>19</td>
<td>854</td>
<td>21</td>
</tr>
<tr>
<td>4</td>
<td>1000</td>
<td>13</td>
<td>986</td>
<td>13</td>
<td>1015</td>
<td>14</td>
</tr>
<tr>
<td>5</td>
<td>934</td>
<td>13</td>
<td>909</td>
<td>14</td>
<td>909</td>
<td>13</td>
</tr>
<tr>
<td>6</td>
<td>933</td>
<td>15</td>
<td>909</td>
<td>15</td>
<td>909</td>
<td>14</td>
</tr>
<tr>
<td>7</td>
<td>933</td>
<td>10</td>
<td>910</td>
<td>10</td>
<td>910</td>
<td>9</td>
</tr>
<tr>
<td>8</td>
<td>1000</td>
<td>13</td>
<td>959</td>
<td>12</td>
<td>986</td>
<td>15</td>
</tr>
<tr>
<td>9</td>
<td>875</td>
<td>19</td>
<td>854</td>
<td>17</td>
<td>875</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Overall GM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>95% Confidence Limits</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GCV</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 1. Results (Lf-eq/ampoule) for 21/230.

Table 3. Stability data from the three-month timepoint with Lf-eq/ml determined by flocculation assay, with n=1 ampoule for each storage temperature.

<table>
<thead>
<tr>
<th>Storage Temperature (°C)</th>
<th>Lf-eq/ml</th>
<th>Relative to -20 °C baseline</th>
<th>Kf time (mins)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-20</td>
<td>1000</td>
<td>-</td>
<td>14</td>
</tr>
<tr>
<td>+4</td>
<td>1000</td>
<td>1.000</td>
<td>14</td>
</tr>
<tr>
<td>+20</td>
<td>1000</td>
<td>1.000</td>
<td>14</td>
</tr>
<tr>
<td>+37</td>
<td>1000</td>
<td>1.000</td>
<td>15</td>
</tr>
<tr>
<td>+45</td>
<td>1000</td>
<td>1.000</td>
<td>20</td>
</tr>
<tr>
<td>+56</td>
<td>824</td>
<td>0.824</td>
<td>23</td>
</tr>
</tbody>
</table>
Table 4. Quantification of tetanus toxoids by flocculation assay in Lf/ml using 21/230 and 66/021 tetanus antitoxins equine reference reagents. Three independent narrow range flocculation assays were conducted for both 21/230 and 66/021 with Kf times in minutes shown in brackets.

<table>
<thead>
<tr>
<th>Assay no.</th>
<th>Tetanus Antitoxin Calibrant</th>
<th>Tetanus Toxoid Quantification by Flocculation (Lf/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Toxoid A</td>
</tr>
<tr>
<td>1</td>
<td>21/230</td>
<td>6090 (34)</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>6090 (30)</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>6090 (31)</td>
</tr>
<tr>
<td>1</td>
<td>66/021</td>
<td>5880 (67)</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>5880 (66)</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>5880 (67)</td>
</tr>
</tbody>
</table>
Appendix A - List of participants (alphabetical order by country)

ARGENTINA

Dr Maria Luisa Brero/ Dr Silvina Gil
Administracion Nacional de Laboratorios e Institutos de Salud (ANLIS)
563 Avenida Velez Sarsfield
Cuidad Autonoma de Buenos Aires

CANADA

Mr Joel Charbonneau
Health Canada
100 Eglantine Driveway
Tunney’s Pasture A/L: 0602A
Ottawa
Ontario

DENMARK

Mr Morten Just Svendsen
AJ Vaccines
Artillerivej 5
Copenhagen
DK-2300

HUNGARY

Mrs Veronika Ondi
GSK Biologicals Kft
Quality Control
Homoki Nagy István utca 1
Gödöllő

INDIA

Dr Arun Bhardwaj
Central Drug Laboratory
CRI Campus
Kasauli
173204

INDIA

Dr Sunil Gairola
Serum Institute of India
212/2, Soli Poonawalla Road
Hadapsar
Pune
411 028

INDONESIA

Mr Dori Ugiyadi
PT. Bio Farma
JL. Pasteur No 28
PO Box 1136
Bandung
40161
THE NETHERLANDS
Dr Amanda Versteilen
Bilthoven Biologicals
Antonie van Leeuwenhoeklaan 9-13
PO Box 457
Bilthoven
3720 AL

UNITED KINGDOM
Dr Shalini Rajagopal
Medicines and Healthcare Regulatory products Agency
Blanche Lane
South Mimms
Potters Bar
EN6 3QG
Appendix B Instructions for Use (IFU)
To whom it may concern

HEALTH CERTIFICATE

We hereby certify that all hens used for the production of Tenax® autogenous, batch No. 746, date of manufacture 24/03/2022, have been in clinical and good health condition and did not show signs of systemic infection disease during a period of 21 days before the day of egg-laying.

Blood samples were taken at the laying examinations and tested negative for infectious anemia, chicken and glandulars at the State Office for Consumer Protection and Food Safety.

The birds are treated with an unvaccinated strain number. Hens with the following antibodies were included in the production of the serum batch 242, 245, 246, 248, 250, 252, 254, 256, 257, 259, 260, 261, 263, 265, 267, 268, 272, 273, 275, 277, 278, 280, 281, 282, 283, 284, 285, 286, 287, 289, 290, 291, 292, 293, 294, 295, 296, 297, 298, 299.

On behalf of

[Signature]

[Name]

(Qualified Person)

National Institute for Biological Standards and Control

Declaration regarding Viral Safety

We hereby confirm that Tenax® autogenous batch No. 746, manufactured from raw serum batch No. 722, complies with the following specifications (for method and duration see Annex 1):

- no pathogenic virus detectable
- hemagglutination-inhibition test: non-reactive
- Immunoperoxidase assay for exclusion of DHV-1, DHV-2 and DHV-3

Testing has been performed by MDR-MPI Fraunhofer-Institut für Mikrobiologische Forschung GmbH, German.

[Signature]

[Name]

[Date]

[Place]

[Institue]