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PHARMEUROPA BIO & SCIENTIFIC NOTES

AUGUST 2024

Collaborative study for the characterisation of the BINACLE Assay for *in vitro* detection of tetanus toxicity in toxoids – Part 1

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Abstract

For several decades the European Pharmacopoeia monographs *Tetanus vaccine (adsorbed) (0452)* and *Tetanus vaccine for veterinary use (0697)* required that *Specific toxicity and Absence of toxin and irreversibility of the toxoid* of each bulk of tetanus toxoids had to be tested by an *in vivo* toxicity test in guinea pigs before it could be included in vaccines for human or veterinary use. In line with the 3Rs concept of replacing, reducing and refining animal experiments, an *in vitro* method for the detection of active tetanus neurotoxin (TeNT) has been developed at the Paul-Ehrlich-Institut (PEI, Germany). This method, the so-called BINACLE (binding and cleavage) assay, uses the receptor-binding and proteolytic properties of TeNT for the specific detection of active toxin molecules. Successful in-house validation studies as well as a small-scale transferability study had demonstrated that this method may represent a suitable alternative to the compendial *in vivo* toxicity test. As a follow up, an international collaborative study aimed at verifying the suitability of the BINACLE assay as a potential alternative to the guinea pig toxicity test for tetanus toxoids was organised by the European Directorate for the Quality of Medicines & HealthCare (EDQM) under the aegis of its Biological Standardisation Programme (BSP). Within the framework of this study, coded BSP136, a feasibility phase – also referred to as Phase 1 – was run to select and qualify critical study reagents and samples and to assess the performance of the BINACLE Standard Operating Procedure developed by the project leaders. Then the international collaborative study aimed at evaluating the BINACLE, referred to as BSP136 Phase 2, was started. A total of 19 international laboratories (comprising vaccine manufacturers as well as national control laboratories) were supplied with a detailed assay protocol, critical reagents required for the assay, three samples consisting of three different bulk tetanus toxoids donated by major European vaccine manufacturers and one international standard toxoid. Each of the participants was asked to perform three independent BINACLE assays following the provided protocol. The statistical analysis of the results showed that most of the participating laboratories were able to perform the BINACLE assay according to the provided protocol. However, the results obtained by the participants varied widely, and not all the laboratories were able to achieve a sensitive detection of active TeNT. Multiple factors may have contributed to the elevated variability of the BSP136 study results. From an analysis of these factors, strategies were developed to help increase the standardisation of the BINACLE assay and obtain more consistent results in a follow-up validation study, BSP 136 Phase 3 (Part 2), for which the experimental phase took place in 2023. The present manuscript summarises the outcome of Phases 1 and 2, which constitute Part 1 of the BSP136 project.

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Keywords

BINACLE, EDQM, European Pharmacopoeia, *in vitro*, tetanus toxin, tetanus vaccine, 3Rs

1. Introduction and background information

Tetanus vaccine production and control standards in the European Pharmacopoeia

Tetanus vaccines for human and veterinary use are produced according to similar methods [1, 2]. In brief, tetanus neurotoxin (TeNT) is produced by a highly toxigenic strain of *Clostridium tetani* (of known origin, history and with a documented and controlled toxicity profile) growing in a liquid medium. Being extracellular, the toxin is accumulated in culture medium which, once the cultivation period is finished, is separated from cells by means of centrifugation and/or filtration. The toxin content (expressed in Lf per millilitre) of the cell supernatant is determined by the Ramon flocculation assay [3, 4]. After that, detoxification of produced TeNT with formaldehyde takes place. The detoxification step may be performed on crude toxin (culture filtrate) or on purified toxin. In both cases, the method used for detoxification is expected to avoid destruction of the immunogenic potency of the toxoid and occurrence of specific toxicity. The bulk purified toxoid thus obtained is tested for sterility and antigenic purity. In addition, the bulk purified toxoid must meet the other requirements of European Pharmacopoeia (Ph. Eur.) monographs *Tetanus vaccine (adsorbed) (0452)* and *Tetanus vaccine for veterinary use (0697)* [5, 6].

When BSP136 started in 2014, the 8th edition of Ph. Eur. monographs *0452* and *0697* [7, 8] in force at that time required that *Specific toxicity and irreversibility of toxoid* were to be demonstrated in addition to the *Absence of toxin* before the bulk toxoid (blended or not with other antigens) was adjuvanted and dispensed into containers and subjected to potency and safety testing on the final bulk or final batch.

The quality control tests described above were mandatory and required the use of large numbers of animals. In Europe, production and quality control of human and veterinary medicines represented about 14 % of the total number of animals used for scientific purposes by that time [9]. As it is commonly recognised that large numbers of animals are used in the toxicity and antigenicity testing of Clostridial vaccines [10], work in this field was given high priority by interested parties, resulting in attempts to develop new *in vitro* methods at manufacturers' quality control (QC) laboratories or at Official Medicines Control Laboratories (OMCLs).

In parallel, the experts of European Pharmacopoeia (Ph. Eur.) Groups 15 (Human Vaccines and Sera) and 15V (Veterinary Vaccines and Sera) challenged the usefulness of some *in vivo* tests in vaccine control and notably the need for testing bulk tetanus vaccine for irreversibility of toxoid. Literature analysis, surveys and collection of batch release data as well as an experimental study addressing the stability of TeNT under the conditions prescribed for the irreversibility test [11] were undertaken and the analysis of the information collected led the groups of experts to propose the revision of the Ph. Eur. monographs on tetanus vaccines to the Ph. Eur. Commission. As a result, the revision of the *Absence of toxin* test specifications and the waiving of the *irreversibility of toxoid* part of the test as well as the removal of the *Specific toxicity* checking requirement were implemented as of 2021 [12, 13]. Currently, only testing for the *Absence of toxin* to assess residual toxicity is required to be performed in guinea pigs and the same procedure – requiring subcutaneous injection of 1 mL of bulk toxoid corresponding to at least 500 Lf into five animals, followed by a 21-day observation period – is required for both human and veterinary vaccine control [5, 6].

Background information on the project

With respect to the 3Rs approach, a new method for the *in vitro* detection of residual active TeNT in tetanus toxoid bulks has been developed at the Paul-Ehrlich-Institut (PEI) [14]. This new method, referred to as "BINACLE assay" for "binding and cleavage assay" (see Annex I – Assay Principle), has been reported as highly specific and sensitive with respect to tetanus toxin detection [15]. It has already been successfully tested in an in-house validation study [16], thus demonstrating that it constitutes a potential alternative to current manufacturers' in-process controls of tetanus toxoid bulks (i.e. *in vivo* toxicity tests). These results were then supplemented by an international transferability study of the BINACLE assay for *in vitro* detection of tetanus toxicity involving four laboratories y [17].

Finally, in 2014, a project coded as BSP136 – divided into Phases 1 and 2 and hereafter referred to as BSP136 Part 1 – was initiated with the aim of examining the features of the BINACLE assay. The principal aim of this study was to examine the applicability of the BINACLE assay as a potential alternative to the mandatory *in vivo* safety tests for *Absence of toxin and irreversibility of toxoid* according to Ph. Eur. monographs *0452* and

0697 [7, 8]. To this end, the precision of the BINACLE assay was to be assessed by means of intra-laboratory variation (repeatability) and inter-laboratory variation (reproducibility) of tetanus toxin quantification results. In addition, the study was expected to allow estimation of the limit of detection (LOD) of the BINACLE assay in each participating laboratory.

Dr Heike Behrensdorf-Nicol and Dr Beate Kraemer were nominated as co-project leaders. This study was co-ordinated by the European Directorate for the Quality of Medicines & HealthCare (EDQM) of the Council of Europe under the aegis of the Biological Standardisation Programme (BSP), a research programme co-sponsored by the Council of Europe and the European Commission, aimed at establishing Ph. Eur. reference preparations and validating new pharmacopoeial methods, and particularly committed to considering promising alternative 3R methods.

Because some toxoids could generate intrinsic background signals in the BINACLE assay [16], prior to the validation study it was necessary to undertake preparatory steps (referred to as Phase 1) aimed at pretesting toxoid batches donated for the study by three European manufacturers (one veterinary and two human vaccine manufacturers). A fixed toxoid concentration of 20 Lf/mL was used for all toxoid-containing test samples during the study. This concentration, which represents a typical final vaccine concentration, was chosen based on the specifications of monograph *0452* in the Ph. Eur. 8th [7] and 9th [18] editions, in force until 2020, which stated that for most of the prescribed toxicity tests, the toxoids must be diluted to the same concentration as in the final vaccine.

Phase 1 was also aimed at pretesting commercially available reagents previously identified as critical, such as TeNT, reference toxoid (WHO 2nd International Standard (WHO 2nd IS) for Tetanus Toxoid for use in Flocculation Test), trisialoganglioside GT1b, a biotin-conjugated secondary antibody and peroxidase-conjugated streptavidin. All critical reagents mentioned above and nine batches of non-adsorbed toxoids from three different manufacturers were thus pretested at PEI and found suitable. Three batches of non-adsorbed toxoids (one from each of the three manufacturers mentioned above) were then selected for inclusion in the upcoming collaborative study. Another special feature of the BINACLE assay is the use of two specialised reagents which are not commercially available, i.e. recombinant synaptobrevin (rSyb), the substrate specifically cleaved by TeNT, and anti-synaptobrevin (anti-Syb), an affinity-purified polyclonal antibody specific for cleaved synaptobrevin. The samples of rSyb and anti-Syb necessary for the performance of the collaborative study were donated by PEI to the EDQM. All the other reagents considered critical were purchased and provided to participants by the EDQM, except for the reference TeNT which was to be purchased by the participants.

Logistics for the sourcing and provision of test samples as well as their shipment to non-European laboratories proved to be difficult. Therefore, the experimental part, i.e. the collaborative study, was started in 2016.

2. Participants

Nineteen laboratories active in the field of human or veterinary vaccine control (seven from the public sector and twelve manufacturer's control laboratories) from eight European countries, Canada, India, Brazil and the United States of America participated in this collaborative study (see Section 6). In this manuscript participants are referred to by code numbers from 1-19, which are not related to the order of listing used in Section 6.

3. Materials and methods

3.1. Materials

Tetanus neurotoxin

A commercial batch corresponding to the reference tetanus toxin (coded TeNT) was reserved centrally for the BSP136 study, and one vial of this batch was purchased by each of the participants from Sigma-Aldrich.

Toxoid samples

Three toxoid samples corresponding to bulk toxoid batches representative of the quality of currently produced products, which had been donated by the respective manufacturers, and a reference toxoid (WHO 2nd IS for Tetanus Toxoid for use in Flocculation Test, 690 Lf/ampoule, NIBSC code 04/150) were tested in the

study. One vial of each of the test toxoids – coded Toxoid A (TdA), Toxoid B (TdB) and Toxoid C (TdC)) – and two ampoules of the WHO 2nd IS for tetanus toxoid were supplied to each study participant.

Critical reagents

Critical reagents such as biotin-SP-conjugated AffiniPure goat anti-rabbit IgG (H+L) (Dianova GmbH, Hamburg), peroxidase-conjugated streptavidin (Dianova GmbH, Hamburg) and trisialoganglioside GT1b (Sigma-Aldrich) were centrally purchased by the EDQM. Two other critical reagents, namely anti-synaptobrevin (affinity-purified, polyclonal antibody specific for cleaved synaptobrevin) and rSyb (stock solution, 12 µM) were provided by the PEI.

All critical reagent batches provided for the study as well as the TeNT batch and tetanus toxoid samples were prequalified at the project leaders' laboratory (PEI).

Materials and non-critical commercial reagents

Commercial reagents considered non-critical were purchased by the participants themselves following directives given in the study protocol (where necessary grade and/or provider and product code were indicated). These reagents comprised asolectin (Sigma), bovine serum albumin (BSA), DC™ Protein Assay Kit II (Bio-Rad), distilled water and various chemicals: 30 % H_2O_2, ethanol, formaldehyde, glycerol, chlorohydric acid, potassium chloride, monopotassium phosphate, methanol, sodium acetate, sodium chloride, sodium hydroxide, disodium phosphate, PIPES (1,4-piperazinediethanesulfonic acid), sucrose, sulphuric acid, tris(2-carboxyethyl)phosphine hydrochloride) (TCEP), trimethylamine *N*-oxide (TMAO), tetramethylbenzidine (TMB) and Tween 20.

Among materials to be provided by the participants themselves, provider and product codes were indicated for MaxiSorp microplates and tubes.

3.2. Methods

3.2.1. BINACLE assay procedure and test design

The BINACLE assay was to be performed in accordance with the Standard Operating Procedure (SOP) given in the study protocol and summarised in Annex II.

To mimic insufficiently detoxified toxoids, three test toxoids (TdA, TdB and TdC) as well as one reference toxoid (WHO 2nd IS) were assayed in each test. Each toxoid was spiked with TeNT at 0.1, 0.5 and 5.0 ng/mL.

Toxicity was measured by analysing the signal that was elicited by each TeNT concentration in each toxoid solution, i.e. the absorbance units (AU) that were recorded on the corresponding wells on each test plate. In addition, several controls were included on each test plate:

- *Blank controls*: wells coated with ganglioside GT1b that were incubated with Binding Buffer (BB) without toxoid and without TeNT, for the detection of the background signal.
- *Negative controls*: wells not coated with ganglioside GT1b that were incubated with BB, with toxoid diluted in BB, or with spiked toxoid diluted in BB, for the detection of non-specific binding.
- *Positive controls*: wells coated with ganglioside GT1b, that were incubated with TeNT diluted in BB at 0.1, 0.5, 5.0 ng/mL, for the detection of TeNT.
- *Toxoid controls*: wells coated with ganglioside GT1b that were incubated with unspiked toxoid diluted in BB, for the detection of possible matrix effects.

The plate layouts used are provided in Annex II (Figure 1 and Figure 2).

The following validity criteria were set for assessment of assay performance:

- *Blank and negative controls*: the signals should not be greater than 1.0 AU.
- *Positive controls*: on each plate, an increase in signals (AU) depending on the TeNT concentration should be visible: a continuous increase in signal intensity from the columns with no TeNT, via columns with low and medium TeNT concentration through to columns with high TeNT concentration should be seen.

3.2.2. Study design

Participants were requested to perform three independent assays. In each assay, the samples were analysed using two different 96-well plates.

The performance of each independent BINACLE assay took 3 days and was performed as follows:

Day 1 - Preparation of buffers and samples, preparation of microplates for the binding and cleavage steps, overnight incubation of samples on two binding plates (BP).

Day 2 - Reduction of bound sample material on BPs, transfer of released light chains from BPs to two cleavage plates (CP), cleavage incubation (6 h), overnight incubation with first antibody.

Day 3 - Incubation with biotinylated secondary antibody, streptavidin-peroxidase and a colorimetric peroxidase substrate, photometric measurement of assay signals.

It was required by the organisers to perform each independent assay within a period of 3 consecutive days. To avoid a potential loss of activity of the reconstituted TeNT, the performance of the complete study protocol (i.e. three consecutive assays) had to be completed within 3 weeks (or 4 weeks, if an assay had to be repeated) starting from the day when the TeNT stock solution was prepared.

3.2.3. Reporting of results to the EDQM

After completion of the three assays, the results of the photometric measurements were provided to the EDQM as copies of the reader printouts (raw data), included in the electronic data centrally provided reporting sheet (see example in Annex II Figure 3) and in the form of a signed authorised copy of the standard reporting sheet (for quality assurance reasons).

Participants were also requested to record in writing and to send to the EDQM information about all unexpected issues and deviations from the test protocol which had occurred during performance of the assays together with the results of the photometric measurements.

3.2.4. Central statistical analysis

An independent central analysis of all the experimental data received by November 2016 was performed at the EDQM. The signals (AU) measured by the laboratories for the different controls (blank, negative and positive controls) as well as for the toxoids unspiked with TeNT were compared using descriptive statistics, e.g. boxplots and tables of medians and interquartile ranges (IQRs). Conformance of these controls to the validity criteria defined in the study protocol (see section 3.2.1.) was also evaluated.

The signal (AU) generated by the toxoid samples (WHO 2nd IS, TdA, TdB and TdC) unspiked and spiked with increasing concentrations of TeNT (0, 0.1, 0.5 and 5 ng/mL) were analysed using two approaches. The first one consisted in calculating an LOD for TeNT for each laboratory, the second in calculating potency estimates for TeNT in the toxoid samples that were used for repeatability and reproducibility assessment.

3.2.4.1. *Calculation of limits of detection for TeNT*

LODs were calculated according to Tholen *et al.* [19].

A cut-off was calculated per preparation – BB, WHO 2nd IS, TdA, TdB and TdC – and by assay, based on the AU of the toxoid controls and of the blank controls using the formula mean + 3.3 × standard deviation. The means and standard deviations were calculated using Huber's robust formulae.

The AU of the BB and of the four test samples (WHO 2nd IS, TdA, TdB, and TdC) spiked with 0.1, 0.5 and 5 ng/mL of TeNT were then compared to the cut-off values for each run. The LOD for each laboratory was determined as the TeNT concentration for which the proportion of AU above cut-off was greater than or equal to 80 % for at least three of the four toxoid samples assayed.

3.2.4.2. *Calculation of potency estimates for the spiked toxoids*

The potency estimates for the four toxoid samples (WHO 2nd IS, TdA, TdB, and TdC) spiked with TeNT were calculated relative to the positive control (TeNT diluted with BB) for each individual assay, using a slope ratio analysis.

The slope ratio analysis was carried out on exponentiated AU of the positive control and toxoid samples spiked with TeNT to improve the linearity of the regression lines. Huber's weights were used to alleviate the effect of possible outlying AU on LODs and potency estimates.

Then, using a one-way random analysis of variance to evaluate the repeatability and reproducibility of the BINACLE assay, the intra- and inter-laboratory variability of the potency estimates was calculated per toxoid. The intra-laboratory variation was calculated for each participant and then was averaged over the 19 laboratories to provide an estimate of the assay repeatability. The sum of the intra-laboratory and inter-laboratory variations represented the assay reproducibility. Calculated values were reported as geometric coefficients of variation (GCV in %).

The descriptive plots, summary statistics and analyses of variance were generated using the R statistical software (R Cran) [20]. The slope ratio analyses were carried out using CombiStats 5.0 [21].

4. Results

4.1. Compliance to the prescribed study schedule, design and assay procedure

Out of the 19 participants, twelve (Laboratories 1, 2, 8, 10-17 and 19), carried out three independent assays within a period of four consecutive weeks maximum, as requested.

Laboratory 4 carried out three assays in three consecutive weeks, plus one assay 6 weeks later using new vials of material (with the exception of the WHO 2nd IS). Other deviations from the testing period are shown in Table 1.

As regards assay numbers, six laboratories carried out four assays in total. Laboratories 4, 6 and 9 carried out a fourth assay as the results of the previous assays were unsatisfactory. Laboratories 5 and 7 obtained unexpectedly low AU for all wells of the second assay. Laboratory 18 obtained unexpectedly high AU for the blank control of the third assay. These laboratories decided to carry out a fourth assay that was used to replace the questionable assay in the statistical analysis.

Table 1 – *Overview of assays performed per laboratory*

Laboratories	Assays performed	Assays reported	Testing period
1, 2, 8, 10, 11, 12, 13, 14, 15, 16, 17, 19	1-2-3	1-2-3	4 weeks
3	1-2-3	1-2-3	5 weeks
4	1-2-3-4	1-2-3-4	3 assays in 3 weeks plus one 6 weeks later
5	1-2-3-4	1-3-4	3 weeks
6	1-2-3-4	1-2-3-4	3 weeks
7	1-2-3-4	1-3-4	4 weeks
9	1-2-3-4	1-2-3-4	3 weeks
18	1-2-3-4	1-2-4	4 weeks
Total	63	60	

As regards the compliance to the BINACLE SOP, several deviations were reported. For example, Laboratory 4 reported that they extended several incubation times in one of their assays, and that they accidentally dropped one microplate after addition of TMB in another test. Laboratory 3 used different BSA products of different purities in each of their assays. Laboratory 12 deliberately used higher toxin concentrations than indicated in the protocol for assays 2 and 3 after having generated low toxin signals in assay 1. Laboratories 8 and 14 reported that they had changed the BSA concentration in the Antibody Buffer during the study or the TMAO batch, respectively.

4.2. Control results

In view of the assessment of the BINACLE assay performance, several validity criteria were set for the blank, negative and positive controls (see section 3.2.1). Nevertheless, all collected data (including those from assays showing blank and negative controls with AU greater than 1.0) were considered in this study to obtain a comprehensive picture of the variability of BINACLE results and to elaborate strategies for reducing this variability in the future.

4.2.1. Blank controls

On each of the 96-well plates assayed, blank controls correspond to five wells that were coated with ganglioside GT1b and where BB without toxoid and toxin was added (see plate layouts in Annex II Figure 1 and Figure 2). These controls reflect the background signal of the assay and the AU recorded should not be greater than 1.0.

A graphical representation of the AU for blank controls is provided in Figure 1A. Each boxplot represents the AU of the assays performed by one laboratory.

Comparison of the AU obtained by the different laboratories revealed that the results differed in terms of both the medians and their dispersion (IQR), indicating that the laboratories obtained significantly different background signals. For example, Laboratory 9 has a median of 0.135 along with an IQR of 0.037, while Laboratory 3 has a median of 1.170, which exceeds the 1.0 threshold prescribed in the validity criteria, along with an IQR of 0.670.

The high IQR of Laboratory 3 is explained by the heterogeneity between the AU of the three assays (centred on 1.498, 0.771 and 1.196). Similar heterogeneity was seen in three additional laboratories (5, 8 and 16).

4.2.2. Negative controls

On each of the 96-well plates assayed, negative controls correspond to the first three rows of the 96-well plate that were not coated with GT1b (see plate layouts in Annex II Figure 1 and Figure 2). These controls reflect non-specific binding and should not result in AU greater than 1.0.

A graphical representation of the AU for negative controls is provided in Figure 1B. The boxes are similar in shape to those shown in Figure 1A, leading to similar conclusions as drawn for the blank controls. That is, the laboratories obtained significantly different non-specific binding signals (ranging from a median absorbance value of 0.139 for Laboratory 9 to a value of 1.237 for Laboratory 3). In addition, six laboratories (3, 5, 7, 8, 16 and 19) got heterogeneous results, reflected by the great height of the boxes or spread of data points (circles) in Figure 1B.

Negative controls are also characterised by a high number of circles located beyond the vertical lines issuing from each box. These circles appear above the upper vertical line in most cases and denote the positive skewness of the data distributions. The rate of such data points ranges from 1 % to 8 % (4 % on average) (Table 1 of Annex III). The corresponding number is stable at low TeNT concentrations (36, 37 and 35 occurrences at 0, 0.1 and 0.5 ng/mL TeNT) and doubles at the 5 ng/mL concentration (68 occurrences) (Table 2 of Annex III). Moreover, these data points are more frequent for the BB (57) and WHO 2nd IS (39) than for the other toxoid samples (maximum of 33 occurrences for toxoid TdA).

4.2.3. Toxoid controls

A graphical representation of the signal generated by unspiked toxoid samples (for each of the four toxoids, five GT1b-coated wells containing the toxoid in BB without TeNT) is provided in Figure 1C. The boxplots are similar in shape to those for blank controls (five GT1b-coated wells containing BB without toxoid) (see Figure 1A), which indicates that the matrix of the four toxoids tested has a limited effect on the signal generated in the assay.

Figure 1A – *Blank controls (wells coated with GT1b, incubated with BB without TeNT)*

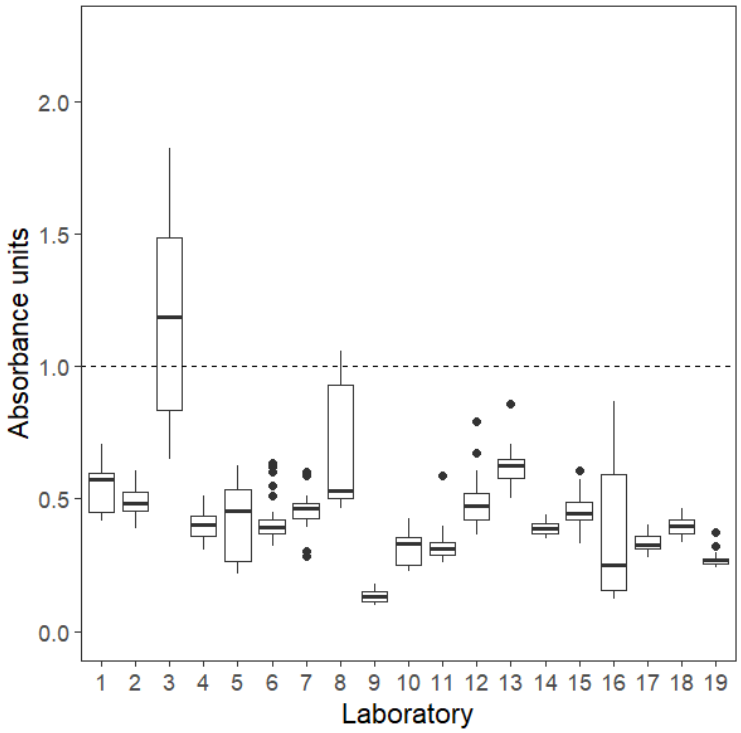


Figure 1B – *Negative controls (wells not coated with GT1b coating and incubated with BB, with toxoid samples diluted in BB or with TeNT-spiked toxoid samples diluted in BB)*

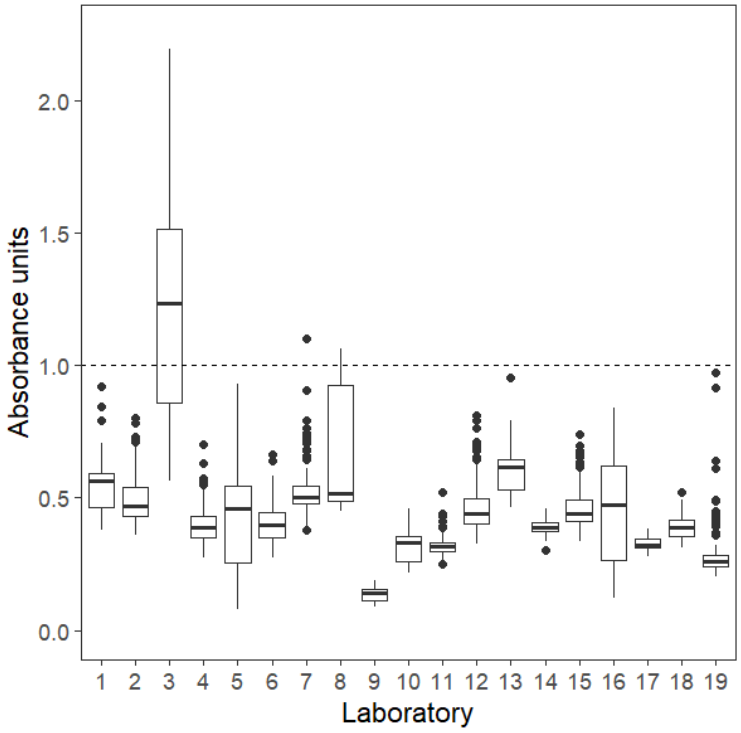
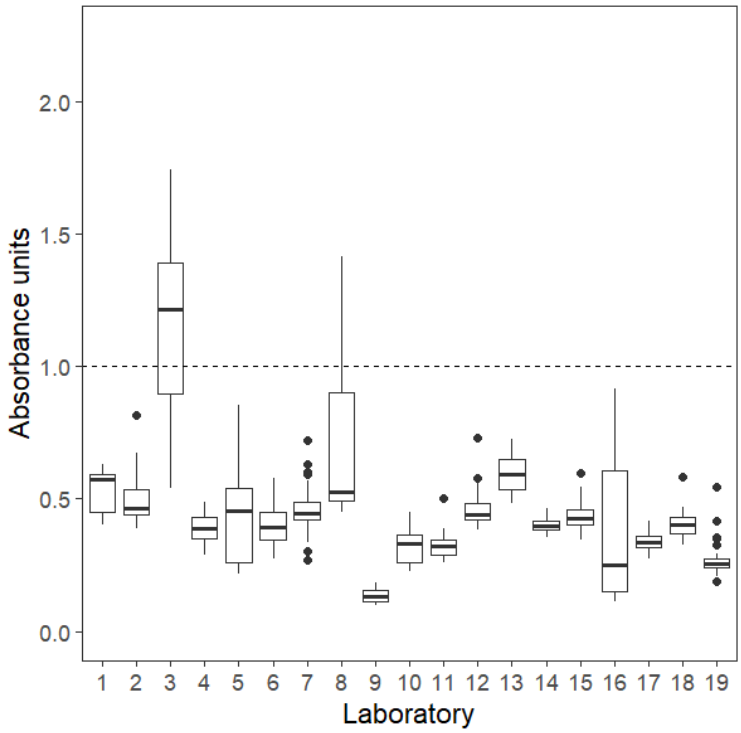


Figure 1C – *Toxoid controls (wells coated with GT1b, incubated with unspiked toxoids diluted with BB)*



Figures 1A-C. Boxplots of absorbance values. Each boxplot represents the AU of the assays performed by a laboratory. The black line in the box interior is the median. The box itself represents the interquartile range (IQR), which encompasses 50 % of the values around the median. The vertical lines issuing from each box represent the whiskers. The positive skewness of the data distribution is illustrated by the data points (represented by circles) located above the upper whisker.

4.2.4. Positive controls

On each of the 96-well plates assayed, positive controls correspond to three series of five GT1b-coated wells that contain TeNT diluted in BB at 0.1, 0.5 or 5 ng/mL, respectively (see plate layouts in Annex II Figure 1 and Figure 2). The protocol required, as an assay validity criterion, that the signal (AU) increases continuously with increasing TeNT concentration.

Table 2 shows the median increase in AU for each laboratory at each TeNT concentration step (Step 1: 0-0.1 ng/mL, Step 2: 0.1-0.5 ng/mL, Step 3: 0.5-5 ng/mL) compared to the corresponding background value. The median values reported in the table are calculated on all assay results for the laboratory and are rounded to the nearest multiple of 0.05 for the sake of clarity. As an example, Laboratory 19 had a background signal centred on 0.25, and the data showed no signal increase at the first two concentration steps. A signal increase was observed at the last concentration step only (plus 0.05 in between 0.5-5 ng/mL TeNT).

In Table 2, the laboratories are sorted from the lowest to highest increase in AU for the concentration step 0.5-5 ng/mL, leading to three categories:

- Category 1: low signal increase (median increase ≤ 0.5): Labs 3, 6, 9, 12, 16 and 19.
- Category 2: moderate signal increase (0.5 < median increase ≤ 1.0): Labs 1, 4, 5, 10, 11 and 17.
- Category 3: high signal increase (median increase > 1.0): Labs 2, 7, 8, 13, 14, 15 and 18.

These three categories reflect a weak, moderate and strong dose-response relationship, respectively. Most of the laboratories belonging to the first two categories show a signal increase (median ~ 0.1) at the second concentration step only. On the contrary, the laboratories belonging to the third category (i.e. strong dose-response relationship) show a signal increase (median = 0.05) at the first concentration step.

Table 2 – *Positive control median increase in signal (AU) per laboratory and by TeNT concentration step (ng/mL)*

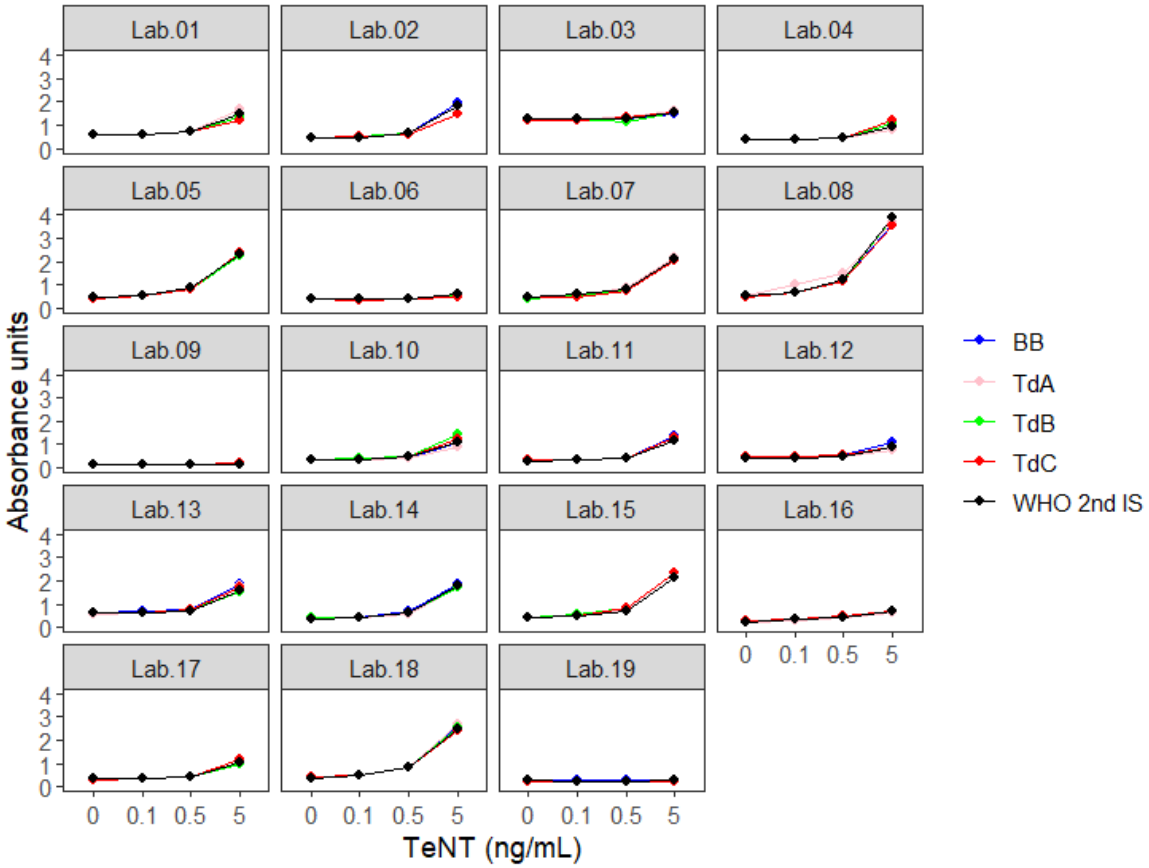
Category (median signal increase)	Lab	Blank ctrl. signal	Positive control median increase in signal		
			Step 1 0-0.1	Step 2 0.1-0.5	Step 3 0.5-5
1 (Low)	9	0.15	0.00	0.00	0.00
	19	0.25	0.00	0.00	0.05
	3	1.20	0.05	0.05	0.20
	16	0.25	0.05	0.10	0.20
	6	0.40	0.00	0.05	0.25
	12	0.45	0.05	0.10	0.45
2 (Moderate)	17	0.35	0.00	0.05	0.60
	4	0.35	0.00	0.05	0.70
	1	0.60	0.00	0.10	0.75
	10	0.35	0.00	0.10	0.75
	11	0.30	0.00	0.10	0.95
	5	0.45	0.10	0.25	1.00
3 (High)	13	0.65	0.05	0.10	1.10
	14	0.40	0.05	0.25	1.20
	2	0.50	0.05	0.15	1.25
	7	0.45	0.05	0.30	1.35
	15	0.45	0.05	0.35	1.50
	18	0.40	0.10	0.35	1.55
	8	0.50	0.10	0.35	2.40

Blank ctrl. signal: median AU of blank controls (five GT1b-coated wells without TeNT).
Positive control signal: median AU of positive controls (five GT1b-coated wells with TeNT diluted in BB for each of three TeNT conc.: 0.1, 0.5, 5 ng/mL).
Median increase: rounded to the nearest multiple of 0.05.
Laboratories: sorted from lowest to highest median signal increase in AU at Step 3, then grouped into three **categories**: 1 (Low), 2 (Moderate), 3 (High) (median increase ≤ 0.5, in between 0.5 and 1.0, > 1.0, respectively).

4.3. Median profiles of signals for TeNT in BB and in toxoids

Median profiles of the signals (AU) obtained for the four TeNT-spiked toxoids tested and for the positive controls (TeNT-spiked BB), obtained in each of the 19 participant laboratories are shown in Figure 2. In most laboratories, the individual median profiles of the four spiked toxoids cannot be differentiated from each other or from the positive control profile due to their strong overlap.

Figure 2 – *Median TeNT signal profiles obtained per laboratory and by sample*



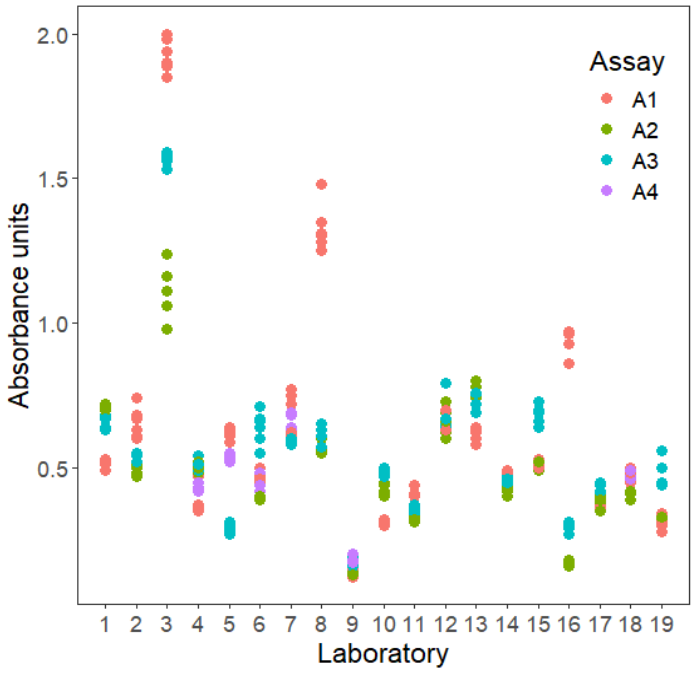
The laboratory code is given above each graph. Sample-Colour code: BB-Blue, TdA-Pink, TdB-Green, TdC-Red, WHO 2nd IS-Black.

4.4. Limits of detection

To determine the LODs, the cut-off values calculated per assay and per preparation for all laboratories shown in Table 3 of Annex III were used. Corresponding cut-off values are shown in Figure 3.

Figure 3 – *Cut-off values (expressed in AU) per laboratory and by assay*

Red-, green-, blue- and violet-coloured circles correspond to Assays 1-4, respectively.



The proportions of AU above cut-off for each laboratory and each toxoid are shown in Table 4 of Annex III.

The study design did not allow precise determination of the actual LOD for all laboratories; therefore, the following approach was used to analyse the data generated. The LOD for each laboratory was determined by convention as the lowest tested TeNT concentration for which the proportion of signals above cut-off was greater than or equal to 80 % for at least three spiked toxoids among WHO 2nd IS, TdA, TdB and TdC. However, as only a limited number of toxin concentration levels was included in the study, it is possible that the actual LOD reached by the individual laboratories was even somewhat lower than the LOD values defined using this approach. Therefore, a TeNT concentration interval in which the actual LOD is included was determined for each laboratory. The LOD and TeNT concentration interval determined for each participant are shown in Table 3.

Eight laboratories had an actual LOD in between 0.5 and 5 ng/mL TeNT, and six laboratories in between 0.1 and 0.5 ng/mL TeNT. Four laboratories had an LOD greater than 5 ng/mL TeNT. One laboratory had an LOD lower than 0.1 ng/mL TeNT: in this laboratory, the proportion of AU above cut-off was 100 % for the four toxoids spiked with 0.1 ng/mL TeNT.

Table 3 – *LODs expressed as TeNT concentration (ng/mL, in parentheses) and as TeNT concentration interval (ng/mL, in brackets) per laboratory*

≤0.1 (0.1)	]0.1, 0.5] (0.5)	]0.5, 5] (5)	> 5 (not known)
Lab 18	Lab 2	Lab 1	Lab 3
	Lab 5	Lab 4	Lab 9
	Lab 7	Lab 6	Lab 16
	Lab 11	Lab 8	Lab 19
	Lab 14	Lab 10	
	Lab 15	Lab 12	
		Lab 13	
		Lab 17	

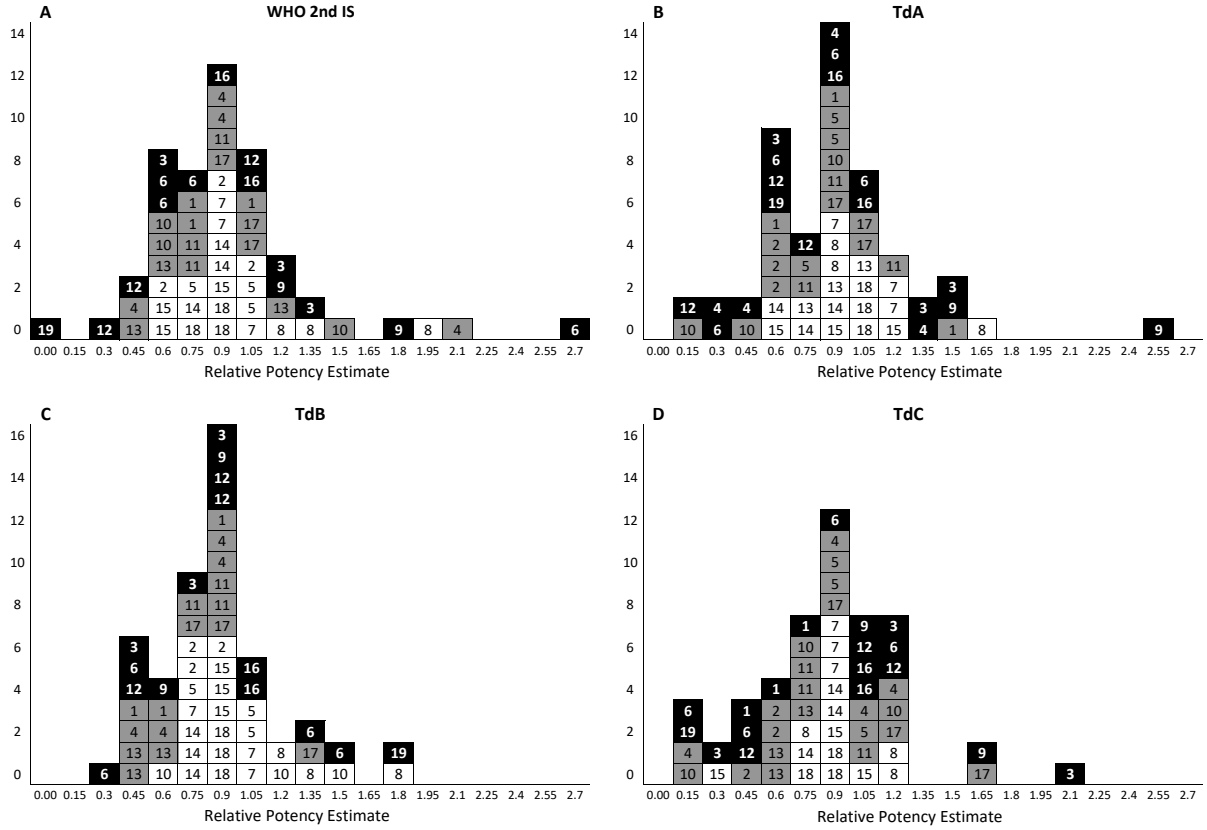
4.5. Repeatability and reproducibility of the BINACLE assay

For the assessment of the repeatability and reproducibility of the BINACLE assay, the potency estimates of the TeNT diluted in WHO 2nd IS, TdA, TdB and TdC were calculated relative to the positive control (TeNT diluted at three concentrations in BB) for the assays performed by each laboratory, to obtain relative potency (RP) estimates.

Table 5 of Annex III shows the RP estimates per assay and per laboratory together with the linearity of regression lines and the precision of RP estimates, which were used as indicators of the quality of the fitted regression model.

Table 4 shows the distribution of the RP estimates of the independent assays performed by the 19 laboratories. The distribution of RP estimates was similar for the four tested toxoids and showed a wide spread. For each test sample, the maximum frequency corresponds to the class [0.9, 1.05[RP, implying most RP values are centred around 1. Importantly, the RP estimates of assays corresponding to strong responses are almost always located in the centre of the histograms, indicating that a strong dose-response relationship is required to generate both precise and homogeneous potency estimates in the BINACLE assay.

Figure 4 – *Histograms of relative potency (RP) estimates of test samples*



A: TeNT spiked into WHO 2nd IS.
B: TeNT spiked into TdA.
C: TeNT spiked into TdB.
D: TeNT spiked into TdC.
The numbers in the boxes represent the laboratory codes.
Colour code: white, grey and black boxes correspond to strong (category 1), moderate (category 2) and weak (category 3) dose-response relationships (as defined in Table 2), respectively.

Table 4 shows the calculated GCV for each test sample and laboratory. The repeatability, expressed as an overall GCV per laboratory (averaged over test samples) is also shown in the table. The repeatability varies over a wide range (from 21 % to 565 %) for the 19 participants, but shows a correlation with the strength of the dose-response relationship (strong, moderate and weak). In other words, a strong dose-response relationship is most likely required to achieve good assay repeatability.

Table 4 – *Repeatability of relative potency (RP) estimates per laboratory and by toxoid*

			GCV (%)				
Category (dose-resp.)	**Lab**	**N RPs***	**WHO 2nd IS**	**TdA**	**TdB**	**TdC**	**Overall**
1 (Weak)	16	2	25	53	1	8	28
	9	2	111	147	96	115	117
	12	3	264	491	123	251	277
	3	3	168	252	130	1 005	349
	6	4	19	187	561	775	364
	19	3	n.a.	n.a.	n.a.	n.a.	n.a.
2 (Moderate)	11	3	32	70	39	55	50
	17	3	34	21	101	124	75
	1	3	62	153	108	85	103
	13	3	272	58	83	56	120
	10	3	201	957	234	673	482
	4	4	218	220	187	2478	565
3 (Strong)	14	3	11	42	9	11	21
	18	3	25	12	16	31	22
	7	3	23	52	38	12	34
	2	3	77	10	22	29	39
	5	3	40	24	67	18	40
	8	3	89	128	81	82	95
	15	3	70	139	14	401	160

***N RPs**: number of relative potency estimates per toxoid.
Repeatability: variability between independent RP estimates obtained by the same laboratory. The repeatability is expressed as a geometric coefficient of variation (GCV in %) and is calculated for each toxoid and on average (Overall).
n.a.: not available, the slope ratio analysis converged for one assay of Laboratory 19. Therefore, no repeatability can be estimated for this laboratory.

The repeatability and reproducibility of RP estimates per test sample are indicated in Tables 5A-C. The results obtained during this analysis are consistent with the observation that not all laboratories had a good command of the BINACLE assay. Indeed, when statistical analysis was first carried out on the complete set of RPs (55 assays run by the 19 laboratories), poor reproducibility was found: the GCVs reported in Table 5A range from 47 % to 88 % (GCV = 68 % on average). In contrast, when RP estimate showing poor precision (either RP estimates with 95 % relative confidence limits greater than 25 %, see Table 5B, or RP estimates with confidence limits greater than 10 %, see Table 5C) were excluded from the analysis, the reproducibility of the BINACLE assay was considerably improved, resulting in an overall GCV of 38 % (Table 5B) and 28 % (Table 5C).

Tables 5A-C – *Repeatability and reproducibility of relative potency (RP) estimates per test sample*

Table 5A – *All RP estimates*

				GCV (%)	
Sample	**N Lab#**	**N RPs***	**Mean RP**	**Repeatability**	**Reproducibility**
WHO 2nd IS	19	55	0.84	67	67
TdA	19	55	0.82	58	66
TdB	19	55	0.83	44	47
TdC	19	55	0.74	88	88
		Overall	0.81	65	68

Table 5B – *RPs estimated with a precision lower than 25 %*

				GCV (%)	
Sample	**N Lab#**	**N RPs***	**Mean RP**	**Repeatability**	**Reproducibility**
WHO 2nd IS	16	48	0.84	41	42
TdA	17	44	0.88	32	34
TdB	16	46	0.85	31	37
TdC	16	44	0.85	30	36
		Overall	0.85	34	38

Table 5C – *RPs estimated with a precision lower than 10 %*

				GCV (%)	
Sample	**N Lab#**	**N RPs***	**Mean RP**	**Repeatability**	**Reproducibility**
WHO 2nd IS	14	35	0.90	23	23
TdA	15	36	0.91	21	27
TdB	15	38	0.88	25	27
TdC	15	36	0.89	20	33
		Overall	0.89	22	28

N Lab: number of laboratoires.
* **N RPs**: number of relative potency estimated per toxoid.
Repeatability: variability between independent assay results obtained by the same laboratory.
Reproducibility: variability between independent assay results obtained by different laboratories.
The repeatability and the reproducibility are expressed as geometric coefficients of variation (GCV in %), including 5A: all assays, 5B: assays where RPs are estimated with a precision lower than 25 %, and 5C: assays where RPs are estimated with a precision lower than 10 %.

The distribution of the mean RPs of the test samples was also influenced by the precision of the individual RP estimates. They range from 0.74 to 0.84 (overall mean of 0.81) when the complete set of RPs is considered (Table 5A). After exclusion of RPs estimated with a precision greater than 10 %, they form a homogeneous group (from 0.88 to 0.91) centred on 0.89 (Table 5C).

As a final step in the analysis, expanded uncertainty was assessed using statistical intervals at ± 2 times the method reproducibility. These intervals were calculated to illustrate the range of possible RP estimates that laboratories could report, in 95 % of cases, for a preparation centred on an RP value equal to 0.90 (i.e. the median RP taken as an example).

As a result of the GCVs calculated above, and considering a preparation of true RP value equal to 0.90, RPs estimated using the BINACLE assay would be, with a 95 % confidence level:

- For a GCV of 68 %: in a range of 0.32 to 2.5.
- For a GCV of 38 %: in a range of 0.48 to 1.7.
- For a GCV of 28 %: in a range of 0.55 to 1.5.

4.6. Analysis of the sources of BINACLE assay variability

An exact root cause analysis for each individual laboratory that experienced difficulties or obtained inconclusive results with the BINACLE assay could not be performed in the context of the study. However, from the comments obtained from the participants during the study, several factors were identified that probably had a marked impact on the study outcome:

- Some participants deliberately introduced changes to the assay conditions or used different batches of reagents in their successive assays. It is very likely that such changes contributed to the elevated inter-assay variability experienced by some of the laboratories.

- Differences between the reagent batches may also be an important factor. The reagents regarded as most critical for assay performance (e.g. antibodies, receptor molecules and substrate proteins) had been centrally supplied to all participants. However, several other reagents and buffers had to be ordered by the participants themselves (e.g. BSA, TMAO, TCEP and TMB). Accordingly, the corresponding reagent batches used by the different laboratories had not been examined for their performance in BINACLE testing beforehand. It cannot be excluded that differences between the reagents used by the participants contributed to the high variability of the results. For example, in-house data obtained at the PEI indicate that a batch of the reagent TMAO which was distributed by a large supplier of laboratory reagents during the study period led to less pronounced signal-enhancing effects in BINACLE assays than usually observed with other TMAO batches. It is therefore possible that laboratories using this TMAO batch generated somewhat lower toxin signals than laboratories using a different batch.
- Regional differences regarding the availability of reagents may also have played a role in the outcome of the study. It is noteworthy that several participants from non-European countries were not able to achieve sensitive toxin detection with the BINACLE assay. One of these laboratories reported difficulties in obtaining several reagents from their local suppliers in the high purity grade that was recommended in the study protocol. For example, a BSA product used in this context was not certified as protease-free, and therefore may have caused degradation processes in the TeNT stock solution, which could account for the weak activity-based toxin signals that were measured.
- All the participants purchased and used the same batch of TeNT, but had to prepare their own TeNT spike solutions by dissolving the commercial lyophilised toxin. This reconstitution process was somewhat complex and may have contributed to variability between the concentrations of the spike solutions used in the different laboratories. Indeed, discrepancies between the expected concentration of the toxin stock solution (based on the manufacturer's specifications) and the actual concentrations calculated from the results of the DC™ protein quantification assay performed were reported. The toxin reconstitution step and the subsequent protein determination were critical and somewhat complicated steps that may have contributed to the variability of the study outcome.
- Working practices could also be a relevant factor. For example, if the microplates are left empty for longer periods of time after the washing procedures, the wells can dry out, which may have a negative effect on the final assay signals, for example by generating high background signals. Or, if the wells are filled very slowly, samples located in certain areas of the microplate can experience longer incubation times than samples in other areas. Accordingly, pipetting technique and the type of equipment used (e.g. multi- versus single-channel pipettes, or automatic microplate washer versus manual washing) could influence the assay outcome.

5. Discussion, conclusions and recommendations

The BSP136 collaborative study was performed to characterise the variability and applicability of the BINACLE assay for the *in vitro* detection and quantification of active TeNT and to evaluate its suitability as an alternative method to the *in vivo* safety/toxicity testing of tetanus toxoids. A total of 19 laboratories from different countries (EU and non-EU) and with different backgrounds (vaccine manufacturers and public sector medicines control laboratories) were included in Part 1 of the study to cover a wide range of interested parties.

To cover a broad spectrum of relevant toxoids, tetanus toxoids from three different European vaccine manufacturers as well as one WHO IS toxoid were included in the study. It has been reported that, depending on the production process, toxoids from different manufacturers differ in their behaviour in the BINACLE assay [15]: specifically, toxoids from several vaccine manufacturers can be subjected to BINACLE testing even at high concentrations, whereas toxoids from some other producers induce high background signals, and are therefore less suitable for the study. Therefore, only toxoids that were prequalified and found suitable for BINACLE testing were then used in this collaborative study. A fixed toxoid concentration of 20 Lf/mL was used for all toxoid-containing test samples during the study. This concentration, which represents a typical final vaccine concentration, was chosen based on the specifications of monograph *0452* in the Ph. Eur. 8th and 9th editions [7, 18], in force until 2020, which stated that for most of the prescribed toxicity tests, the toxoids must be diluted to the same concentration as in the final vaccine.

In the absence of access to insufficiently detoxified toxoid production batches, toxoid samples that had been spiked with active TeNT to mimic insufficiently detoxified material were included in the study in order to

characterise the detection limit of the BINACLE assay. However, concerns in setting a suitable quantification limit by the BINACLE were identified in relation to the following facts: (i) no reliable data concerning the detection limit of the tetanus toxicity test in guinea pigs could be found in the literature; (ii) at the time of the study start, the various toxoid safety tests prescribed in the Ph. Eur. [7, 8] for human and veterinary tetanus vaccines differed strongly with regard to the respective animal weights (between 250 and 450 g) and injection volumes (between 1 and 5 mL), implying that it was difficult to define a unitary value for the detection limit of the guinea pig test; (iii) the specific toxicity of tetanus toxin preparations, even if obtained from the same supplier, may vary between batches, and no internationally accepted reference toxin preparations are available. However, from in-house data generated at the PEI, it has been estimated that the detection limit of the animal test is probably in the range between 0.1 and 0.9 ng/mL for TeNT [15]. With reference to this estimation, spike concentrations of 0.1, 0.5 and 5 ng/mL TeNT were chosen for the study. The two lower spike concentrations fall within the range of the potential *in vivo* detection limit (with 0.1 ng/mL representing the lower boundary of this range), whereas the highest spike dose was chosen as it was expected to induce a clear response in the animal test.

The experimental part of the BSP136 Part 1 study showed that most of the participating laboratories were able to perform the BINACLE assay according to the provided protocol and a close analysis of the data generated and presented herein allows the BINACLE to be considered as a promising alternative to the *in vivo* tetanus toxin test.

Out of the 19 participating laboratories:

- seven laboratories achieved a very sensitive detection of active TeNT: six laboratories had a detection limit between 0.1 and 0.5 ng/mL TeNT, which corresponds to the estimated range for the *in vivo* detection limit, and one laboratory showed a very low LOD below or equal to 0.1 ng/mL TeNT. Among these laboratories, six had never performed the method before and only one had already been acquainted with the BINACLE assay due to participation in a former assay transferability study [16];
- eight laboratories achieved a somewhat less sensitive detection of active TeNT, i.e. an LOD in between 1 and 5 ng/mL TeNT. Among these laboratories, six had never performed the method before and two had already been acquainted with the BINACLE assay due to participation in a former assay transferability study [16];
- four laboratories that had never performed the method before did not manage to properly detect TeNT and had an LOD greater than 5 ng/mL. Among these laboratories, one did not observe any toxin-induced signal increases at all.

However, the observed LODs covered a wide range of TeNT concentrations (50-fold min.), which suggests there is a need for better standardisation of the assay.

As regards the variability of the BINACLE assay observed in this study, the following observations could be made. The repeatability values that were calculated based on the RP estimates varied widely between the laboratories, i.e. from 21 % GCV to 565 % GCV. Laboratories that obtained comparatively strong dose-response relationships usually showed better repeatability values than laboratories with moderate or flat dose-response curves. The high variability of the study results is also reflected by a high overall GCV value obtained for the reproducibility (68 %). However, when focusing on the RPs determined with good precision (i.e. with 95 % confidence limits lower than 10 %), a strongly improved reproducibility was found (28 % GCV).

The variability data as well as the LOD results equally indicated that, in order to achieve the reliable application of the BINACLE assay in the future, it was very important to identify strategies for enhancing the standardisation of the method.

However, the analysis of the factors that contributed to the high variability of the results and the unsatisfactory assays results in some of the laboratories was not straightforward, as the BINACLE assay consists of several successive steps and comprises the use of many reagents. Whenever unwanted effects (like high variability, high background values or low specific toxin signals) occurred, they may either have been caused by reagent- or equipment-related issues, or by irregularities in the performance of the assays – or by a combination of these factors. Nevertheless, several targets for further improvement of the BINACLE protocol could be identified based on the Part 1 study results.

In conclusion, despite its shortcomings, this study demonstrated the suitability of the BINACLE assay as a potential alternative to the guinea pig toxicity test for tetanus toxoids. The results from some of the participants

showed that the BINACLE method can be properly implemented rapidly and performed in a repeatable way without encountering major problems. Moreover, the study demonstrated that, when performed properly, the method allows sensitive detection of active TeNT in the range between 0.1 and 0.5 ng/mL.

Based on the results obtained in the present study, an extension of BSP136 was recommended to allow the full inter-laboratory validation of the BINACLE assay for the *in vitro* detection of tetanus toxicity in toxoids to be achieved.

The advice of the project leaders provided on the proposed follow-up study design is summarised hereafter.

To simplify and optimise the study design, and prevent the need to process several microtiter plates in parallel:

- Only one prequalified tetanus toxoid batch should be selected as test sample, the TeNT concentration range should be adapted to allow better determination of LODs, and more precise instructions and specifications should be given in the study protocol.
- As regards the selection and provision of batches of reagents, more reagents that were found critical should be centrally purchased, prequalified and distributed to the participants to ensure that reagents of appropriate quality are used in all laboratories.
- In addition, more ready-to-use solutions (e.g. commercial TMB solution, commercial 10× PBS stock solution) should be used to avoid some of the complex steps for the preparation of stock and working solutions.
- Aliquots of ready-to-use TeNT, biotin-conjugated goat anti-rabbit IgG, peroxidase-conjugated streptavidin, ganglioside GT1b, TMAO, BSA, TCEP and asolectin should be provided.
- Considering the logistical difficulties for the sourcing and provision of test samples and of standardised commercial reagents, it was also recommended to run the study with a core group (up to 10 laboratories) including as a priority European manufacturers' QC laboratories.
- A workshop with the study participants should be organised ahead of the experimental part, and during the study, proactive and extended technical support should be offered to participants in case of difficulties encountered during the assay performance.

This strategy was endorsed by the BSP steering committee and the experts of Groups 15 and 15V of the Ph. Eur., and the preparatory steps for the follow-up study were undertaken. Originally referred to as BSP136 Phase 3 and constituting Part 2 of the BSP136 project, the follow-up study was launched in February 2023 and successfully completed in December 2023. The study outcome has been published in a separate manuscript [22].

6. **List of participants** (in alphabetical order by country)

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W. QUINTILIO, Instituto Butantan, Brazil
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7. Acknowledgements

The study organisers wish to thank all participants for their valuable participation in this study. Heike Behrensdorf-Nicol and Beate Krämer are gratefully acknowledged for being project leaders and also for performing the experiments for the feasibility studies and supporting the EDQM in material procurement and shipment. PEI technicians Ursula Bonifas and Jolanta Klimek are also warmly acknowledged for their active involvement in performance of the experimental work and their excellent support work throughout the study. PEI is gratefully acknowledged for the donation of reagents. The EDQM is grateful to AJ Vaccines A/S (formerly SSI), Merial (now part of Boehringer-Ingelheim) and Sanofi Pasteur for the donation of the starting materials for the study.

This study was run within the framework of the Biological Standardisation Programme, a research programme run under the aegis of the Council of Europe and jointly sponsored by the European Commission (project code BSP136). Marie-Emmanuelle Behr-Gross and Natalia Sinitiskaya coordinated the project.

Statistical analysis was completed by David Le Tallec. Sally Woodward provided expert organisational support as well as editorial and secretarial assistance in the preparation of the protocol and the report. Louise Birrell and Alice Eleuterio are acknowledged for their support in the linguistic editing and layout work on this manuscript. This document was produced with financial assistance by the European Union. The views expressed herein can in no way be taken to reflect the official opinion of the European Union.

8. Abbreviations

AU: absorbance expressed in absorbance units; anti-Syb: anti-synaptobrevin; BB: binding buffer; BP: binding plate; BSA: bovine serum albumin; BSP: Biological Standardisation Programme; CP: cleavage plate; EDQM: European Directorate for the Quality of Medicines & HealthCare; EU: European Union; GCV: geometric coefficient of variation; GT1b: trisialoganglioside GT1b; IgG: immunoglobulin G; IS: International Standard; IQR: interquartile range; LOD: limit of detection; NIBSC: National Institute for Biological Standards and Control; OMCL: Official Medicines Control Laboratory; PEI: Paul-Ehrlich-Institut; Ph. Eur.: European Pharmacopoeia; RP: relative potency; rSyb: recombinant synaptobrevin; RT: room temperature; SOP: Standard Operating Procedure; TCEP: tris(2-carboxyethyl)phosphine hydrochloride; Td: toxoid; TeNT: tetanus neurotoxin; TMAO: trimethylamine N-oxide; TMB: 3,3´,5,5´-tetramethylbenzidine; WHO: World Health Organization.

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22. Behrensdorf-Nicol HA, Le Tallec D, Sinitskaya N *et al.* Collaborative study for the characterisation of the BINACLE Assay for *in vitro* detection of tetanus toxicity in toxoids – Part 2. *Pharmeur Bio Sci Notes* 2024;2024:162-92.

10. Annexes

Annex I – Assay Principle

Annex II – BINACLE procedure for BSP136 Part 1

Annex III – Additional statistical data

Annex I – Assay Principle

TeNT consists of two protein subunits: the heavy chain mediates the receptor binding and uptake by target cells, and the light chain is a protease which specifically cleaves the protein synaptobrevin inside the target cells.

For the BINACLE assay, ganglioside GT1b (the receptor for TeNT) is immobilised on a microplate and incubated with the test samples. Unbound sample molecules (i.e. molecules lacking a functional binding domain) are removed by washing, and bound molecules are treated with a reducing agent in order to release and activate the toxin light chain. The supernatant with the activated light chains is then transferred to a second microplate containing immobilised rSyb. In the presence of active TeNT light chains, synaptobrevin is cleaved, and the cleavage fragment is detected using a cleavage site-specific polyclonal antibody followed by a biotinylated secondary antibody, peroxidase-conjugated streptavidin and a peroxidase substrate. The resulting colorimetric signal is measured. By taking into account the specific binding and cleavage capacities of TeNT, the BINACLE assay is able to detect functional TeNT molecules more reliably than other *in vitro* methods.

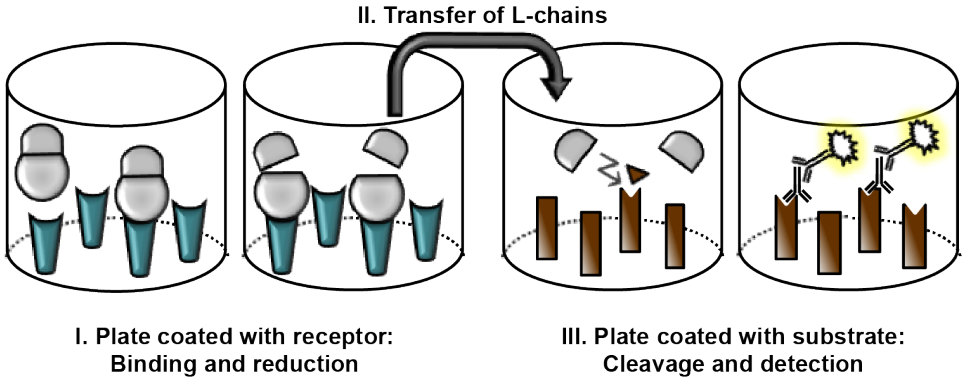


Figure adapted from: Behrensdorf-Nicol H, Weisser K, Krämer B. *ALTEX* 2015;32:41-6.
Schematic overview of the BINACLE assay for *in vitro* detection of active tetanus neurotoxin:
(I.) TeNT molecules (grey) bind to immobilised receptors (green). Upon reduction, the proteolytic L-chains of TeNT are released and activated.
(II.) The supernatant with the L-chains is transferred to a plate coated with the substrate protein rSyb (brown).
(III.) The L-chains cleave the rSyb, and the cleavage product is finally detected using antibodies.

Annex II – BINACLE procedure for BSP136 Part 1

Each BINACLE assay was to be performed on three consecutive working days. Tasks were organised according to a detailed SOP, which is summarised as follows.

NB: Where no procedure is given for the generation of stock solutions, it is assumed that they were provided as such by the study organisers or that they correspond to solutions prepared according to the manufacturers' instructions for commercial products.

Day 0: Preparation of stock solutions for WHO reference toxoid, asolectin and TeNT reference toxin

1. Preparation of the WHO reference toxoid stock solution

Two vials of WHO 2nd IS for Tetanus Toxoid for use in Flocculation Test (690 Lf/ampoule, NIBSC code 04/150) were reconstituted in 0.9 % sodium chloride solution at a final concentration of 1 000 Lf/mL. The resulting reference toxoid stock solution from both vials was transferred into a sterile tube and stored at 4 °C.

2. Preparation of asolectin stock solution

Asolectin was diluted at a final concentration of 40 mg/mL in PBS pH 7.1 and the resulting solution was sonicated in a way allowing a transparent solution to be obtained. The resulting asolectin stock solution was aliquoted and stored at -20 °C.

3. Preparation of the TeNT stock solution and determination of the protein concentration by the DC™ Protein Assay

The content of one TeNT vial was reconstituted in 200 µL sterile aqua bidest. The resulting solution was immediately tested by the DC™ Protein Assay (Bio-Rad) using a procedure derived from the standard "Microplate Assay Protocol" described in the DC Protein Assay Instruction Manual provided by the manufacturer. The protein concentration of the reconstituted TeNT solution was calculated with the help of the BSA standard curve obtained by linear regression. The residual reconstituted TeNT solution was mixed with the same volume of sterile BSA solution (10 mg/mL) and the resulting TeNT stock solution was stored at 4 °C. The concentration of the TeNT stock solution was calculated by dividing by 2 the protein concentration that was determined for the reconstituted TeNT.

Day 1: Preparation of microplates and binding step

4. Preparation of buffers, TMB stock solution and GT1b working solution

- **Blocking Buffer**: a buffer containing PBS pH 7.1 / 0.5 % BSA / 5 % sucrose / 100 µg/mL asolectin was prepared using the asolectin stock solution generated on day 0. The Blocking Buffer was stored at RT until use. The remaining material from the thawed aliquot of the asolectin stock solution was stored at 4 °C for use on day 2.
- **Binding Buffer (BB)**: 100 mM PIPES / 150 mM NaCl, pH 6.4 / 1 % BSA was prepared and stored at RT until use.
- **Wash Buffer**: PBS (w/o Ca2+, Mg2+) pH 7.1 / 0.05 % Tween 20 was prepared and stored at RT during the assay.
- **TMB stock solution**: TMB solution at 6 mg/mL in ethanol was prepared and stored at 4 °C for up to 1 week.
- **GT1b working solution**: GT1b stock solution (1 mg/mL in methanol) was diluted in ethanol at a final concentration of 0.04 mg/mL. The resulting GT1b working solution was stored at RT until use. The remaining GT1b stock solution was stored at -20 °C for use in the following assays.

5. Preparation of the microplates for TeNT binding

- **Coating the binding plates (BP) with ganglioside GT1b**

  Two MaxiSorp microplates (BP1 and BP2) were prepared as follows.

  In each BP, 50 µL of ethanol was added to all wells in rows A, B and C (negative control wells) and 50 µL of the GT1b working solution was added to all wells in rows D to H. The plates were placed for about 2.5 hours in the running laminar flow until the solvent had completely evaporated and the wells looked absolutely dry.

- **Washing the BPs**

  GT1b-coated BPs were washed 4 times with Wash Buffer and excess liquid was removed by tapping the plates on absorbent paper vigorously after the final wash.

- **Blocking of residual protein binding sites**

  250 µL of Blocking Buffer was added to each well of the BPs prior to sealing with adhesive foil and incubation in a microplate thermoshaker for 2 h at 37 °C and 250 rpm.

6. Preparation of TeNT and toxoid samples

- **Preparation of toxoid working solutions**

  The tetanus toxoid stock solutions, i.e. WHO reference toxoid (WHO 2nd IS, 1000 Lf/mL), toxoid A (TdA, 3150 Lf/mL), toxoid B (TdB, 5000 Lf/mL), and toxoid C (TdC, 878 Lf/mL) were diluted in BB to generate working solutions at a final concentration of 20 Lf/mL. The remaining toxoid stock solutions were stored at 4 °C for use in the following assays.

- **Dilution of TeNT in BB, WHO 2nd IS, TdA, TdB or TdC working solutions**

  A TeNT solution of 1 µg/mL was prepared by diluting the TeNT stock solution with BB. From this pre-dilution, five series of four dilutions of TeNT at final concentrations of 5, 0.5, 0.1 and 0 ng/mL of tetanus toxin were prepared using either BB, WHO 2nd IS, TdA, TdB or TdC working solutions as the diluent.

7. Binding of TeNT to the microplates

In order to avoid desiccation of the wells, it was recommended to process BP1 and BP2 in this step successively (not in parallel).

- **Washing**

  The GT1b-coated and BSA-blocked BP1 was washed 4 times with Wash Buffer. After the final wash, all excess liquid was removed.

- **Binding of TeNT**

  100 µL of the samples (TeNT diluted in BB or in tetanus toxoid working solutions) was added to the appropriate wells using the proper plate scheme (see Figure 1). BP1 was sealed with adhesive foil and incubated overnight at 4 °C without shaking.

  BP2 was processed according to the same procedure using the proper plate scheme (see Figure 2).

8. Preparation of the microplates for synaptobrevin cleavage

- **Preparation of synaptobrevin working solution**

  Three vials of 1 mL of rSyb solution at 12 µM were thawed, mixed and used to obtain a synaptobrevin working solution at a final concentration of 1.2 µM in PBS pH 7.1. The residual synaptobrevin stock solution was stored at -70 °C, in case an additional test was required.

- **Coating of the cleavage plates (CP) with synaptobrevin**

  Two MaxiSorp microplates (CP1 and CP2) were prepared as follows.

In each well, 100 µL of the synaptobrevin working solution was added, prior to sealing with adhesive foil and incubation for 2 h at 37 °C, 250 rpm.

- **Blocking of residual protein binding sites**

CP1 and CP2 were inverted and tapped on absorbent paper to remove all excess liquid. 250 µL of Blocking Buffer was added to each well prior to sealing with adhesive foil and incubation overnight at 4 °C without shaking.

Day 2: Reduction and cleavage step

9. Reduction of the bound TeNT

- **Preparation of Reduction Buffer**

PIPES Buffer (30 mL) containing sucrose (1.5 g), asolectin (150 µL of stock solution) and TCEP (150 µL of TCEP stock solution 0.5 M; pH 6.8) was prepared and supplemented with TMAO (1 M final concentration).

- **Preparation of plates**

BP1, BP2, CP1 and CP2 were taken out of the refrigerator and warmed to RT.

Residual liquid was carefully removed from BP1 and BP2 prior to washing 4 times with Wash Buffer. Excess liquid was removed before the plates were washed once with PIPES Buffer and removal of all excess liquid performed. 100 µL Reduction Buffer was added to each well of BP1 and BP2, prior to sealing with adhesive foil and incubation for exactly 30 minutes in a microplate thermoshaker at 37 °C and 250 rpm.

During this incubation time, cleavage plates CP1 and CP2 were washed 4 times with Wash Buffer and excess liquid was removed before the plates were washed once with PIPES Buffer. To prevent excessive drying of the wells, the PIPES Buffer from the extra wash in CP1 and CP2 was only removed shortly before the end of the BP1 and BP2 reduction incubation period.

10. Transfer of supernatants from BP to CP and cleavage of synaptobrevin

The reduced supernatants in the wells in row A of BP1 were mixed by pipetting up and down once with a multichannel pipette, and then the supernatants were transferred to the corresponding wells in row A of the washed CP1. This procedure was repeated accordingly for row B and all other rows of the microplate, without changing pipette tips (in order to save time). Then the pipette tips were changed, and the procedure repeated accordingly for the supernatants from all wells of BP2, which were thus transferred to the corresponding wells of CP2.

BP1 and BP2 were discarded while CP1 and CP2 were sealed with adhesive foil and incubated for 6 hours in a microplate thermoshaker at 37 °C and 250 rpm.

11. Detection of cleaved synaptobrevin

- **Preparation of Antibody Buffer**

PBS pH 7.1 / 0.5 % BSA was prepared and stored at RT until use.

- **Preparation of anti-synaptobrevin antibody working solution**

Using an aseptic procedure, the stock solution of the anti-synaptobrevin antibody was used to generate a 1:2 000 dilution in Antibody Buffer. The remaining stock solution of the anti-synaptobrevin antibody was kept at 4 °C for use in the other assays.

- **Preparation of the cleavage plates**

CP1 and CP2 were washed 4 times with Wash Buffer. After the final wash, all excess liquid was removed and 100 µL of the diluted anti-synaptobrevin antibody was added to each well of the microplates, prior to sealing with adhesive foil and overnight incubation at 4 °C without shaking.

Day 3: Detection of bound anti-synaptobrevin

12. Incubation with biotin-SP-conjugated goat anti-rabbit IgG

- **Preparation of biotin-SP-conjugated AffiniPure goat anti-rabbit IgG working solution**

  The stock solution of the biotin-SP-conjugated AffiniPure goat anti-rabbit IgG (secondary antibody) was used to prepare a 1:1250 dilution in Antibody Buffer. The remaining stock solution of the secondary antibody was stored at -20 °C for use in the following assays.

- **Preparation of plates**

  CP1 and CP2 were washed 4 times with Wash Buffer. After the final wash, all excess liquid was removed and 100 µL of the secondary antibody dilution was added to each well of CP1 and CP2, prior to sealing with adhesive foil and incubation for 45 minutes on a microplate shaker at RT with 150-250 rpm.

13. Incubation with peroxidase-conjugated streptavidin

- **Preparation of peroxidase-conjugated streptavidin diluted solution**

  Peroxidase-conjugated streptavidin stock solution was diluted at 1:4000 in Antibody Buffer. The remaining peroxidase-conjugated streptavidin stock solution was stored at -20 °C for use in the following assays.

- **Preparation of plates**

  CP1 and CP2 were washed 4 times with Wash Buffer. After the final wash, all excess liquid was removed and 100 µL of the diluted peroxidase-conjugated streptavidin was added to each well of CP1 and CP2 prior to sealing with adhesive foil and incubation for 45 minutes on a microplate shaker at RT with 150-250 rpm.

14. Incubation with TMB

- **Preparation of TMB working solution**

  501 µL of TMB stock solution (6 mg/mL, prepared on day 1) was diluted in 27 mL *aqua bidest*, plus 3 mL sodium acetate buffer (1.1 M, pH 5.5) plus 6 µL H_2O_2 (30 %). The resulting solution was protected from light.

- **Preparation of plates**

  CP1 and CP2 were washed 5 times with Wash Buffer. After the final wash, all excess liquid was removed and 100 µL of the TMB working solution was added to each well. The plates were incubated for **exactly** 25 minutes at RT in the dark without shaking. The reaction was stopped by adding 50 µL/well of 1 M H_2SO_4.

15. Reading

- The plates were placed in a photometer and absorbance was read at 450 nm versus 620 nm as reference wavelength.
- Raw data were printed, and the results of the measurement were entered into the Excel result sheet provided by the EDQM (see example in Annex 2).

16. Assay validity criteria

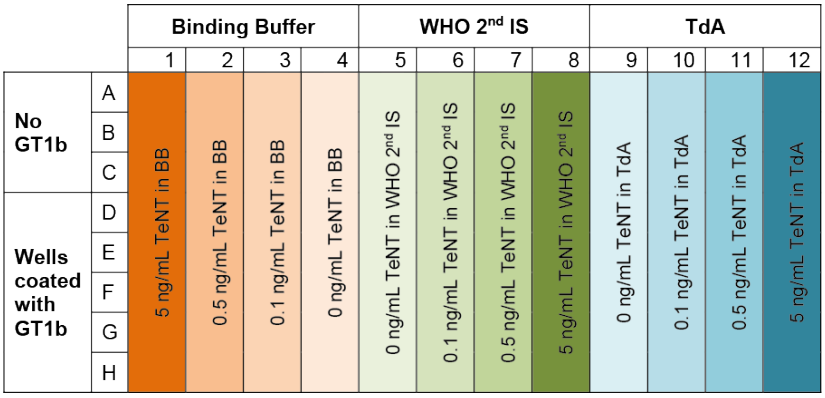
After each assay, checking of the following criteria as indicators for a successful assay performance was required:

- On each plate, an increase in the absorption signals depending on the TeNT concentration should be visible for the positive control (i.e. the TeNT diluted in BB): when looking at the wells in rows D to H, a continuous increase in signal intensity from column 4 (no TeNT, blank control) via columns 3 (low TeNT concentration) and 2 (medium TeNT concentration) through to column 1 (high TeNT concentration) should be seen.
- No extremely high background signals (e.g. AU above 1.0) should occur in the blank controls or in the wells not coated with GT1b.

If these criteria were not fulfilled, it was recommended to contact the project leaders of the BSP 136 study before proceeding with the next assay.

Plate layouts

Figure 1 – *Layout for binding plate 1 (BP1)*



Overview of control wells on BP1

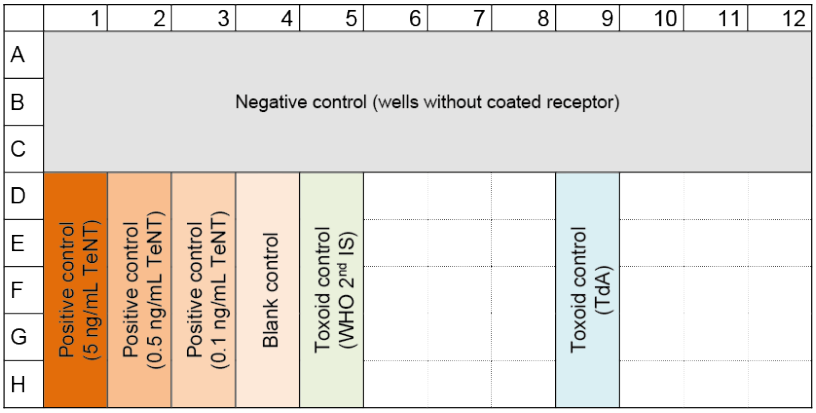
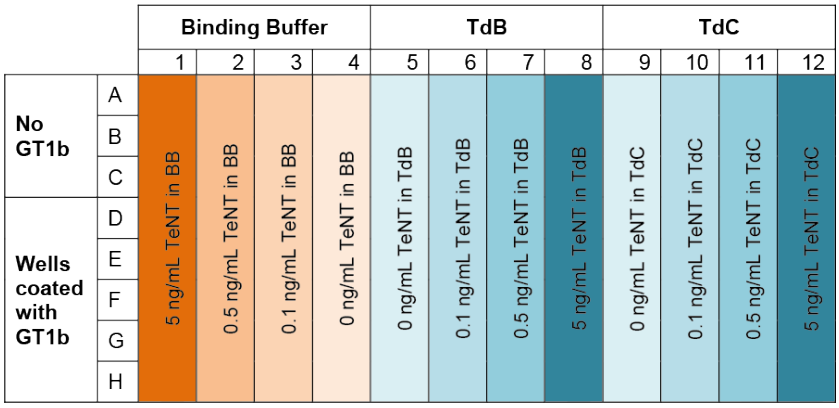


Figure 2 – *Layout for binding plate 2 (BP2)*



Overview of control wells on BP2

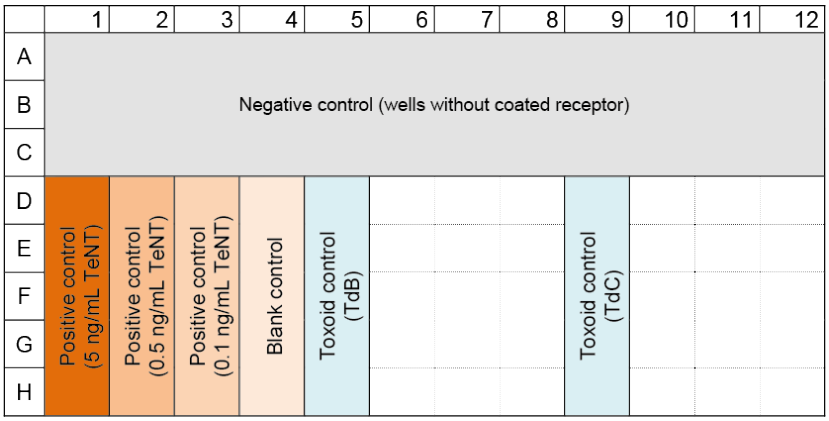


Figure 3 – *BSP136 Data reporting sheet example*

Important: All coloured cells must be completed. Any deviation from the protocol, or anomalies should be reported in the remarks box

General information:

Laboratory:	
e-mail:	
Operator:	

Explanations (see protocol for more details):
Buf = Buffer. Ref = WHO 2nd IS. A, B and C are the test toxoids .
The results of the plate readings should preferably be reported seperately at 450 nm and at 620nm. If your photometer automatically performs the subtraction of the reference measurements and does not allow you to obtain the measurements at both wavelengths separately, you can leave the upper part of this sheet empty and report only the differences in the bottom part.
Do not remove blank plates from the sheet because that will complicate the automatic extraction of data for the analysis.

General design plate 1:

Sample	Buf	Buf	Buf	Buf	Ref	Ref	Ref	Ref	A	A	A	A
Tube	1	2	3	4	8	7	6	5	12	11	10	9
	1	2	3	4	5	6	7	8	9	10	11	12
A	5ng	0.5ng	0.1ng	0ng	0ng	0.1ng	0.5ng	5ng	0ng	0.1ng	0.5ng	5ng
B	5ng	0.5ng	0.1ng	0ng	0ng	0.1ng	0.5ng	5ng	0ng	0.1ng	0.5ng	5ng
C	5ng	0.5ng	0.1ng	0ng	0ng	0.1ng	0.5ng	5ng	0ng	0.1ng	0.5ng	5ng
D	5ng	0.5ng	0.1ng	0ng	0ng	0.1ng	0.5ng	5ng	0ng	0.1ng	0.5ng	5ng
E	5ng	0.5ng	0.1ng	0ng	0ng	0.1ng	0.5ng	5ng	0ng	0.1ng	0.5ng	5ng
F	5ng	0.5ng	0.1ng	0ng	0ng	0.1ng	0.5ng	5ng	0ng	0.1ng	0.5ng	5ng
G	5ng	0.5ng	0.1ng	0ng	0ng	0.1ng	0.5ng	5ng	0ng	0.1ng	0.5ng	5ng
H	5ng	0.5ng	0.1ng	0ng	0ng	0.1ng	0.5ng	5ng	0ng	0.1ng	0.5ng	5ng

General design plate 2:

Sample	Buf	Buf	Buf	Buf	B	B	B	B	C	C	C	C
Tube	1	2	3	4	16	15	14	13	20	19	18	17
	1	2	3	4	5	6	7	8	9	10	11	12
A	5ng	0.5ng	0.1ng	0ng	0ng	0.1ng	0.5ng	5ng	0ng	0.1ng	0.5ng	5ng
B	5ng	0.5ng	0.1ng	0ng	0ng	0.1ng	0.5ng	5ng	0ng	0.1ng	0.5ng	5ng
C	5ng	0.5ng	0.1ng	0ng	0ng	0.1ng	0.5ng	5ng	0ng	0.1ng	0.5ng	5ng
D	5ng	0.5ng	0.1ng	0ng	0ng	0.1ng	0.5ng	5ng	0ng	0.1ng	0.5ng	5ng
E	5ng	0.5ng	0.1ng	0ng	0ng	0.1ng	0.5ng	5ng	0ng	0.1ng	0.5ng	5ng
F	5ng	0.5ng	0.1ng	0ng	0ng	0.1ng	0.5ng	5ng	0ng	0.1ng	0.5ng	5ng
G	5ng	0.5ng	0.1ng	0ng	0ng	0.1ng	0.5ng	5ng	0ng	0.1ng	0.5ng	5ng
H	5ng	0.5ng	0.1ng	0ng	0ng	0.1ng	0.5ng	5ng	0ng	0.1ng	0.5ng	5ng

Assay 1 Plate 1 Date: Wavelength 450 nm

Sample	Buf	Buf	Buf	Buf	Ref	Ref	Ref	Ref	A	A	A	A
Tube	1	2	3	4	8	7	6	5	12	11	10	9
	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Assay 1 Plate 2 Date: Wavelength 450 nm

Sample	Buf	Buf	Buf	Buf	B	B	B	B	C	C	C	C
Tube	1	2	3	4	16	15	14	13	20	19	18	17
	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Assay 1 Plate 1 Date: Wavelength 620nm

Sample	Buf	Buf	Buf	Buf	Ref	Ref	Ref	Ref	A	A	A	A
Tube	1	2	3	4	8	7	6	5	12	11	10	9
	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Assay 1 Plate 2 Date: Wavelength 620nm

Sample	Buf	Buf	Buf	Buf	B	B	B	B	C	C	C	C
Tube	1	2	3	4	16	15	14	13	20	19	18	17
	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Assay 1 Plate 1 Date: Wavelength 450-620nm

Sample	Buf	Buf	Buf	Buf	Ref	Ref	Ref	Ref	A	A	A	A
Tube	1	2	3	4	8	7	6	5	12	11	10	9
	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Assay 1 Plate 2 Date: Wavelength 450-620nm

Sample	Buf	Buf	Buf	Buf	B	B	B	B	C	C	C	C
Tube	1	2	3	4	16	15	14	13	20	19	18	17
	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Remark:

Annex III – Additional statistical data

Table 1 – *Negative control: number and percentage of data points above the upper whisker of boxplots per laboratory*

Laboratory	Number of values		% data points
	Total	N data points	
1	216	7	3 %
2	216	9	4 %
3	210	17	8 %
4	288	10	3 %
5	216	16	7 %
6	288	6	2 %
7	216	16	7 %
8	216	9	4 %
9	288	10	3 %
10	216	11	5 %
11	216	10	5 %
12	216	4	2 %
13	216	11	5 %
14	216	6	3 %
15	216	7	3 %
16	216	7	3 %
17	216	2	1 %
18	216	6	3 %
19	216	12	6 %

Table 2 – *Negative control: number of data points above the upper whisker of boxplots per sample and by TeNT concentration*

Sample	TeNT concentration (ng/mL)				N data points
	0	0.1	0.5	5	
BB	14	13	7	23	57
WHO 2nd IS	11	5	8	15	39
TdA	5	9	8	11	33
TdB	3	4	4	11	22
TdC	3	6	8	8	25
Total/Conc.	36	37	35	68	176

Tables 1 and 2. **Negative controls**: 36 wells that were not coated with GT1b, per plate and per assay. Total = Numbers of negative controls per laboratory (example: 36 wells x 2 plates x 3 assays = 216 negative control voalues for laboratory 1).
N data points: Number of data points located above the upper whisker of box plots.

Table 3 – *Cut-off values (absorbance) per laboratory, by assay and by sample*
Laboratories 1 to 10

		Plate 1			Plate 2		
Lab	**Assay**	**BB**	**WHO 2nd IS**	**TdA**	**BB**	**TdB**	**TdC**
1	1	0.51	0.49	0.52	0.52	0.53	0.52
1	2	0.71	0.70	0.72	0.72	0.71	0.70
1	3	0.63	0.64	0.64	0.67	0.68	0.67
2	1	0.67	0.68	0.74	0.60	0.61	0.63
2	2	0.47	0.47	0.48	0.54	0.50	0.51
2	3	0.55	0.54	0.54	0.54	0.55	0.52
3	1	1.94	1.90	1.89	2.00	1.98	1.85
3	2	1.06	0.98	1.11	1.16	1.06	1.24
3	3	1.57	1.58	1.59	1.56	1.53	1.53
4	1	0.48	0.49	0.47	0.37	0.35	0.36
4	2	0.52	0.52	0.51	0.52	0.48	0.50
4	3	0.54	0.52	0.54	0.48	0.49	0.51
4	4	0.43	0.42	0.43	0.48	0.45	0.43
5	1	0.63	0.64	0.62	0.59	0.59	0.61
5	3	0.30	0.31	0.29	0.27	0.27	0.28
5	4	0.54	0.55	0.54	0.54	0.53	0.52
6	1	0.50	0.47	0.48	0.46	0.45	0.46
6	2	0.44	0.42	0.40	0.44	0.40	0.39
6	3	0.55	0.60	0.64	0.71	0.67	0.66
6	4	0.46	0.46	0.42	0.45	0.48	0.44
7	1	0.72	0.75	0.77	0.60	0.61	0.62
7	3	0.62	0.58	0.59	0.62	0.60	0.60
7	4	0.69	0.64	0.68	0.63	0.62	0.68
8	1	1.30	1.25	1.28	1.35	1.48	1.31
8	2	0.60	0.57	0.56	0.55	0.57	0.55
8	3	0.63	0.65	0.63	0.61	0.61	0.57
9	1	0.15	0.15	0.15	0.12	0.12	0.13
9	2	0.13	0.14	0.13	0.13	0.13	0.13
9	3	0.19	0.18	0.19	0.16	0.17	0.18
9	4	0.19	0.19	0.20	0.17	0.17	0.18
10	1	0.30	0.31	0.31	0.31	0.32	0.31
10	2	0.41	0.40	0.42	0.45	0.44	0.44
10	3	0.47	0.50	0.48	0.49	0.48	0.47

Table 3 (cont.) – *Cut-off values (absorbance) per laboratory, by assay and by sampl*
Laboratories 11 to 19

		Plate 1			Plate 2		
Lab	**Assay**	**BB**	**WHO 2nd IS**	**TdA**	**BB**	**TdB**	**TdC**
11	1	0.40	0.41	0.40	0.41	0.41	0.44
11	2	0.32	0.33	0.33	0.32	0.32	0.31
11	3	0.34	0.33	0.34	0.35	0.36	0.37
12	1	0.63	0.62	0.62	0.66	0.70	0.63
12	2	0.73	0.69	0.60	0.64	0.62	0.66
12	3	0.79	0.65	0.65	0.64	0.66	0.67
13	1	0.63	0.62	0.58	0.64	0.64	0.60
13	2	0.80	0.80	0.69	0.76	0.74	0.78
13	3	0.75	0.76	0.69	0.75	0.72	0.76
14	1	0.48	0.49	0.49	0.46	0.49	0.45
14	2	0.40	0.43	0.42	0.42	0.43	0.42
14	3	0.43	0.44	0.47	0.45	0.46	0.45
15	1	0.52	0.53	0.51	0.53	0.52	0.50
15	2	0.49	0.49	0.50	0.52	0.50	0.52
15	3	0.73	0.66	0.69	0.70	0.66	0.64
16	1	0.86	0.86	0.86	0.96	0.93	0.97
16	2	0.16	0.17	0.16	0.18	0.18	0.16
16	3	0.27	0.29	0.27	0.30	0.31	0.31
17	1	0.36	0.37	0.36	0.37	0.36	0.35
17	2	0.42	0.41	0.40	0.38	0.39	0.35
17	3	0.39	0.41	0.42	0.44	0.44	0.45
18	1	0.46	0.45	0.45	0.49	0.50	0.48
18	2	0.41	0.41	0.39	0.41	0.42	0.41
18	4	0.46	0.46	0.49	0.46	0.47	0.49
19	1	0.34	0.34	0.31	0.31	0.30	0.28
19	2	0.32	0.32	0.32	0.33	0.33	0.33
19	3	0.50	0.56	0.44	0.45	0.44	0.45

Table 4 – *Proportion of absorbance values above cut-off per laboratory by TeNT concentration (mg/mL) and by sample*
Laboratories 1 to 10

Lab	TeNT	BB	WHO 2nd IS	TdA	TdB	TdC
1	0.1	30	40	33	33	20
	0.5	60	73	67	60	67
	5	100	100	100	100	100
2	0.1	23	33	33	27	13
	0.5	100	100	100	100	100
	5	100	100	100	100	100
3	0.1	0	0	0	0	0
	0.5	7	13	0	0	0
	5	37	53	53	13	40
4	0.1	0	0	5	10	5
	0.5	46	30	45	40	30
	5	97	95	95	90	75
5	0.1	77	60	53	60	87
	0.5	100	100	100	100	100
	5	100	100	100	100	100
6	0.1	0	0	10	5	0
	0.5	0	0	5	5	5
	5	93	95	70	80	75
7	0.1	0	27	0	7	0
	0.5	87	93	100	87	80
	5	100	100	100	100	100
8	0.1	63	40	53	60	67
	0.5	80	100	100	73	87
	5	100	100	100	100	100
9	0.1	2	12	16	4	16
	0.5	12	16	32	20	25
	5	68	56	72	72	83
10	0.1	3	0	7	0	0
	0.5	52	53	53	67	50
	5	100	100	87	100	100

Table 4 (cont.) – *Proportion of absorbance values above cut-off per laboratory by TeNT concentration (mg/mL) and by sample*
Laboratories 11 to 19

Lab	TeNT	BB	WHO 2nd IS	TdA	TdB	TdC
11	0.1	7	7	0	27	13
	0.5	100	100	100	100	100
	5	100	100	100	100	100
12	0.1	7	0	0	0	0
	0.5	13	0	0	0	0
	5	100	100	87	100	100
13	0.1	0	0	0	13	0
	0.5	70	27	93	67	33
	5	100	100	100	100	100
14	0.1	80	40	20	27	47
	0.5	100	100	100	100	100
	5	100	100	100	100	100
15	0.1	23	27	33	47	33
	0.5	87	93	87	93	100
	5	100	100	100	100	100
16	0.1	67	33	67	67	67
	0.5	67	67	67	67	67
	5	67	67	67	67	67
17	0.1	10	0	7	0	33
	0.5	73	73	73	93	80
	5	100	100	100	100	100
18	**0.1**	100	100	87	100	100
	0.5	100	100	100	100	100
	5	100	100	100	100	100
19	0.1	3	0	0	0	0
	0.5	0	0	0	7	0
	5	13	0	7	20	0

Taking Laboratory 1 as an example: 40 % of the signals (AU) of the WHO 2nd IS spiked with 0.1 ng/mL TeNT are above cut-off.
Shaded cells: proposed LOD, i.e. TeNT concentration for which the proportion of AU above cut-off is greater than or equal to 80 % for at least three toxoid samples among the four assayed (WHO 2nd IS, TdA, TdB and TdC).

Table 5 – *Relative potency estimates of TeNT per laboratory and by assay*
Laboratories 1 to 9

			Relative potency estimates of TeNT				
Lab.	**Assay**	**Non-Linearity**	**WHO 2nd IS**	**TdA**	**TdB**	**TdC**	**Precision**
1	1	NS	0.705	0.895	0.564	0.652	1-1-1-1
1	2	NS	1.000	1.438	0.474	0.456	1-1-1-1
1	3	NS	0.685	0.644	0.879	0.769	1-1-1-1
2	1	NS	0.990	0.558	0.723	0.488	1-1-1-1
2	2	NS	0.905	0.534	0.804	0.532	1-1-1-1
2	3	NS	0.620	0.582	0.856	0.607	1-1-1-1
3	1	NS	0.611	0.567	0.453	0.280	3-3-3-3
3	2	NS	1.273	1.410	0.889	2.126	3-3-3-3
3	3	NS	1.294	1.513	0.796	1.191	3-3-3-3
4	1	NS	0.836	0.519	0.901	0.841	1-2-1-1
4	2	NS	2.116	0.898	0.407	0.090	2-2-3-3
4	3	NS	0.954	1.424	0.899	1.233	1-1-1-1
4	4	NS	0.386	0.289	0.639	1.108	2-3-2-2
5	1	S	1.005	0.883	0.990	0.898	1-1-1-1
5	3	S	0.977	0.803	1.047	1.002	1-1-1-1
5	4	NS	0.770	0.969	0.694	0.878	1-1-1-1
6	1	NS	0.644	0.352	0.248	0.173	2-3-3-3
6	2	NS	0.642	0.648	0.517	0.463	2-2-2-2
6	3	NS	0.732	0.862	1.277	1.141	2-2-2-2
6	4	S	2.650	1.082	1.560	0.947	3-3-3-3
7	1	NS	1.062	1.213	0.985	0.910	1-1-1-1
7	3	NS	0.897	0.876	0.984	0.866	1-1-1-1
7	4	NS	0.924	1.192	0.773	0.955	1-1-1-1
8	1	S	1.288	0.933	1.341	1.266	1-1-1-1
8	2	NS	1.155	0.872	1.125	0.808	1-1-1-2
8	3	NS	1.947	1.675	1.871	1.264	2-2-2-2
9	1	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
9	2	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
9	3	NS	1.153	1.478	0.893	1.695	3-2-3-3
9	4	NS	1.822	2.576	0.591	1.059	3-3-3-3

Table 5 (cont.) – *Relative potency estimates of TeNT per laboratory and by assay*
Laboratories 10 to 19

			Relative potency estimates of TeNT				
Lab	**Assay**	**Non-Linearity**	**WHO 2nd IS**	**TdA**	**TdB**	**TdC**	**Precision**
10	1	NS	0.589	0.121	0.567	0.209	2-3-1-3
10	2	NS	1.426	0.905	1.568	1.182	1-1-1-1
10	3	NS	0.667	0.468	1.164	0.696	2-2-1-2
11	1	NS	0.864	0.854	0.950	0.755	1-1-1-1
11	2	NS	0.777	1.140	0.905	1.080	1-1-1-1
11	3	NS	0.678	0.723	0.726	0.806	1-1-1-1
12	1	NS	1.020	0.610	0.927	1.134	2-3-2-2
12	2	NS	0.523	0.803	0.497	0.427	2-1-2-2
12	3	NS	0.335	0.188	0.887	1.055	2-3-1-1
13	1	NS	1.234	1.044	0.468	0.549	1-1-2-1
13	2	NS	0.407	0.702	0.388	0.571	2-1-2-1
13	3	NS	0.563	0.870	0.651	0.779	1-1-1-1
14	1	NS	0.894	0.657	0.763	0.813	1-1-1-1
14	2	NS	0.818	0.814	0.789	0.863	1-1-1-1
14	3	NS	0.847	0.882	0.730	0.889	1-1-1-1
15	1	NS	0.956	0.886	0.903	0.833	1-1-1-1
15	2	NS	0.624	1.162	0.869	1.079	1-1-1-1
15	3	NS	0.664	0.551	0.971	0.288	2-2-1-3
16	1	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
16	2	S	1.004	0.826	1.004	0.985	1-1-1-1
16	3	S	0.875	1.072	0.997	1.031	1-1-1-1
17	1	NS	0.869	1.122	1.410	1.718	1-1-1-1
17	2	NS	1.041	1.022	0.800	1.257	1-1-1-1
17	3	NS	1.107	0.952	0.876	0.853	1-1-1-1
18	1	NS	0.945	1.086	0.951	0.766	1-1-1-1
18	2	NS	0.942	1.045	0.871	0.953	1-1-1-1
18	4	NS	0.798	0.983	0.836	0.914	1-1-1-1
19	1	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
19	2	NS	0.075	0.639	1.815	0.090	3-3-3-3
19	3	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

Non-Linearity: the slope ratio analysis is valid if the regression lines of blank controls and test samples are linear. Deviation from linearity can be significant (S) or not (NS).
Precision WHO 2nd IS-TdA-TdB-TdC: half-width of the 95 % confidence interval of the RP estimate with coded values: 1: ≤ 10 %, 2: ≤ 25 %, 3: > 25 %.
n.a.: not available, i.e. the slope ratio analysis failed to converge (no potency estimation) due to no/weak dose-signal relationship.
Colour code: white, grey and black boxes correspond to laboratories with a strong, moderate and weak dose-response.

***Pharmeuropa Bio & Scientific Notes* is published by**

European Directorate for the Quality of Medicines & HealthCare (EDQM)
Council of Europe
7 allée Kastner
CS 30026, F-67081 Strasbourg
France

ISSN: 2075-2504
https://pbiosn.edqm.eu/