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# Collaborative study for the characterisation of the BINACLE Assay for *in vitro* detection of tetanus toxicity in toxoids – Part 2

**H. Behrensdorf-Nicol[1], D. Le Tallec[2], N. Sinitskaya[2], M-E. Behr-Gross[2], C. Göngrich[2]**

## Abstract

Tetanus vaccines for human and veterinary use are produced by formaldehyde-induced inactivation of tetanus neurotoxin (TeNT) purified from *Clostridium tetani* cultures. Due to the high morbidity caused by exposure to TeNT it is essential that the quality control of tetanus vaccines includes testing for absence of tetanus toxin as prescribed by European Pharmacopoeia monographs *0452* and *0697*. Currently this test is carried out in guinea pigs for each bulk of tetanus toxoid. To test the applicability of the *in vitro* BINACLE ("binding and cleavage") assay as an alternative method for the quality control of tetanus vaccines, two collaborative studies were run by the European Directorate for the Quality of Medicines & HealthCare under the aegis of the Biological Standardisation Programme. The first collaborative study indicated that the method allows sensitive TeNT detection. However, a clear conclusion could not be drawn due to the high variability of the results. To address the variability, the protocol was optimised and further standardised for the second study. The study results demonstrated good assay precision, both with respect to repeatability and reproducibility. Importantly, the limit of detection was 0.11 ng/mL TeNT in five out of nine laboratories and 0.33 ng/mL in four out of nine laboratories, suggesting that the BINACLE assay can detect TeNT with similar sensitivity as *in vivo* toxicity tests and can thus be taken into consideration as an alternative method to the current compendial *in vivo* test.

## Keywords

tetanus vaccine, BINACLE assay, *in vitro*, 3Rs, quality control, tetanus neurotoxin

## 1. Introduction

Tetanus vaccines are produced by detoxification of tetanus neurotoxin (TeNT) derived from *Clostridium tetani* cultures. Following detoxification of TeNT using formaldehyde, all tetanus toxoid bulks must undergo a safety test to ensure the absence of active TeNT. This mandatory test is carried out in guinea pigs according to European Pharmacopoeia (Ph. Eur.) monographs *0452 Tetanus vaccine (adsorbed)* or *0697 Tetanus vaccine for veterinary use* [1, 2]. However, the detection limit of this test is not well defined and only scant published experimental evidence is available [3, 4].

The need to replace animal-based safety tests has long been recognised in Europe and the application of the 3Rs principles for the quality control of medicines is enshrined in European legislation [5]. Functional *in vitro* safety tests for toxoid-containing vaccines, such as diphtheria vaccines [6, 7] or *Clostridium septicum*

1 H. Behrensdorf-Nicol. Paul-Ehrlich-Institut, Paul-Ehrlich-Strasse 51-59, D-63225 Langen, Germany.

2 D. Le Tallec, N. Sinitskaya, M-E. Behr-Gross, C. Göngrich (corresponding author's e-mail: christina.goengrich@edqm.eu). European Directorate for the Quality of Medicines & HealthCare (EDQM), Department of Biological Standardisation, OMCL Network & HealthCare (DBO), Council of Europe, Strasbourg, France.

vaccines [8-10], were developed several years ago using cell culture systems. The development of a functional safety test for the quality control of tetanus vaccines has proven to be more difficult, as TeNT does not elicit a cytopathic effect but instead blocks the release of neurotransmitters from its target neurons.

TeNT consists of a heavy and a light chain, which are connected by disulphide bridges. While the heavy chain mediates the receptor binding and uptake of the toxin by the target cells, the light chain, which is released from the heavy chain and activated under the reducing conditions of the intracellular environment, cleaves the synaptic vesicle protein synaptobrevin at a specific amino acid position [11].

The BINACLE ("binding and cleavage") assay for detection of active TeNT takes advantage of this sequential mechanism of action of the toxin: the receptor for TeNT, the ganglioside GT1b, is immobilised on a microplate and incubated with the test samples. Toxin molecules lacking a functional receptor-binding domain are removed by washing, and bound toxin molecules are treated with a reducing agent in order to release and activate their light chain. The supernatant containing the activated light chains is then transferred to a second microplate coated with recombinant synaptobrevin. 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. The method was extensively characterised, and the transferability of the assay was successfully shown in a study with four participants [12-14]. During method development, it became evident that not all toxoids were equally suitable for safety testing using the BINACLE assay in its current form. Despite having passed the Ph. Eur. tests for absence of tetanus toxin (Ph. Eur. *0452* and *0697*), some toxoids induced a high background signal in the assay, thus masking any potential signal derived from low concentration TeNT spikes. However, for several other toxoids sensitive TeNT detection was demonstrated in these early characterisation studies [13].

To examine the applicability of the BINACLE assay for the safety testing of tetanus toxoids as a potential alternative to the mandatory safety test in guinea pigs, a project was initiated by the EDQM within the framework of the Biological Standardisation Programme (BSP, project code BSP136). Dr Beate Krämer and Dr Heike Behrensdorf-Nicol (Paul-Ehrlich-Institut) kindly accepted the role of scientific project leader.

In the first collaborative study 19 laboratories from the public and private sectors from eight European countries, Brazil, Canada, India and the United States of America participated. The goal of the study was to characterise the limit of detection (LOD) as well as repeatability and reproducibility of the BINACLE assay. Toxoids from routine productions of three manufacturers of vaccines for human and veterinary use, as well as the WHO 2nd International Standard Tetanus Toxoid for use in Flocculation Test, were selected for the study and the results showed that the BINACLE assay allowed detection of TeNT spikes in these toxoids with similar sensitivity. However, there were large differences between the results of different participants regarding both detection limit and assay precision [15]. Based on these outcomes no conclusion could be drawn regarding the applicability of the assay as an alternative to the compendial *in vivo* toxicity test. However, they suggested that further method standardisation and optimisation with respect to reagents and study protocol could improve the investigated method parameters. Therefore, a second collaborative study was initiated in which the majority of reagents were prequalified by the project leader, most reagents were provided as ready-to-use solutions, and a streamlined assay protocol was used.

This publication describes the results obtained in the second collaborative study of the BSP136 project and discusses factors that were found to be critical for detection of low amounts of TeNT. A possible implementation strategy and the potential application of the BINACLE method as an *in vitro* alternative to the current *in vivo* test for *Absence of tetanus toxin* according to Ph. Eur. monographs *0452* and *0697* are discussed.

## 2. Participants

Eight manufacturer's control laboratories from six European countries and North America, as well as the laboratory of the project leader, participated in the study. In this report, the participants are referred to by code numbers which are unrelated to the order of their listing in section 9.

# 3. Materials and Methods

## 3.1. Materials

The complete list of reagents, samples and equipment used during the collaborative study is provided in Annex I: the reagents and samples which were prequalified for the assay in the laboratory of the project leader and centrally provided to the participants are listed in Annex I, Table 1, whereas the reagents, materials and equipment provided by the participants are listed in Annex I, Table 2, Table 3 and Table 4.

For future use of the BINACLE method, these lists are indicative only and reagents of equal grade can be used after validation. Recombinant synaptobrevin-2 (rSyb) was contract-manufactured following a protocol developed by the Paul-Ehrlich-Institut (Annex III).

## 3.2. Methods

### 3.2.1. The BINACLE assay

The BINACLE assay was carried out according to the standard operating procedure (SOP) provided in Annex II.

Briefly, a binding plate was coated with ganglioside GT1b. All samples and controls were added to this plate as indicated in the layout for the binding plate (Annex II) and incubated overnight at 4 °C to allow for toxin binding. In parallel, the cleavage plate was coated with rSyb overnight at 4 °C. On day 2 the binding plate was treated with a reducing agent and the resulting supernatant containing the light chains of TeNT was transferred to the cleavage plate. Subsequent to an incubation at 37 °C for 6 hours, cleavage was detected by an overnight incubation with a polyclonal antibody specific to cleaved synaptobrevin followed by incubation with a biotin-conjugated secondary antibody and peroxidase-conjugated streptavidin. The assay signal was developed using 3,3',5,5´-tetramethylbenzidine (TMB) as peroxidase substrate and measured at 450 nm; the reference wavelength was 620 nm.

### 3.2.2. Samples and controls

To mimic insufficiently detoxified toxoids, a toxoid was spiked with TeNT at five different final concentrations (0.11, 0.33, 1.0, 3.0, 9.0 ng/mL TeNT). The toxoid, representative of a production batch of bulk tetanus toxoid that had passed the compendial test for *Absence of tetanus toxin*, was diluted to a typical vaccine concentration (20 Lf/mL final concentration on the plate) for the assay. TeNT dilutions with the same final concentrations as listed above were also prepared in binding buffer without toxoid (positive controls). Background signal was monitored by inclusion of blank control solutions containing either buffer or toxoid without added TeNT. Each sample or control solution was analysed in six replicate wells coated with ganglioside GT1b as receptor. To control for non-specific binding, each solution was additionally incubated in two replicate wells that did not contain ganglioside GT1b (negative controls). The plate layout can be found in Annex II. Participants were instructed to check predefined acceptance criteria (Annex II, SOP) after each assay run to detect any possible problems with the method performance, and to contact the project team before proceeding to the next step if an assay did not meet the criteria.

### 3.2.3. Design of the collaborative study and reporting of results

Participants were requested to perform four independent BINACLE assays using a fresh set of TeNT samples for each of them. The concentrations of the TeNT samples were unknown to the participants.

Each assay, consisting of one binding and one cleavage plate, was to be carried out over a period of three consecutive days (Annex II). To reduce the risk of handling errors, participants were discouraged from carrying out more than one assay per week. Participants were encouraged to contact the project team in case of any questions, planned protocol deviations or problems.

Since several reagents were provided as diluted formulations, it was agreed that one participant would carry out two additional assays in order to assess the stability of the provided solutions over the course of the experimental phase.

After the completion of the four BINACLE assays, the participants sent their results to the EDQM using an electronic data reporting sheet that was provided with the protocol. Participants were requested to provide

the results as raw data, i.e. the AU (absorbance units) recorded at the reference wavelength (620 nm) and at the measurement wavelength (450 nm) for all requested BINACLE assays, either separately for each wavelength or after automatic wavelength correction. Any protocol deviations or unexpected issues were to be noted on the reporting sheets.

### 3.2.4. Additional BINACLE tests with concentrated toxoid

Using the toxoid and the TeNT that was used in the collaborative study, additional assays were run to assess the performance of the BINACLE assay when toxoid concentrations up to 500 Lf/mL are tested. The method was performed according to the SOP (Annex II). Each TeNT spike concentration (0.11, 0.33, 1.0, 3.0, 9.0 ng/mL TeNT final concentration) was diluted in each of the toxoid solutions (20, 100 and 500 Lf/mL toxoid) and in buffer only and tested in triplicate on each plate. Three assays were performed.

### 3.2.5. Central statistical analysis

A central statistical analysis of all data submitted by the participants was carried out at the EDQM. In a first step, the reference wavelength measurements were checked for abnormally high values and the corresponding wells were excluded from further analysis. Then the absorbance values obtained for the reference wavelength (620 nm) were subtracted from the absorbance values obtained for the measurement wavelength (450 nm) for each single well to correct for unspecific signal. The photometer used by Laboratory 7 performed this step automatically.

The resulting corrected absorbance values were explored and summarised using descriptive statistics. Each assay was evaluated based on the criteria laid out in the SOP (Annex II).

The analysis of the intra- and inter-laboratory variability was carried out based on the variability of the relative potency (RP) estimates. To obtain the RP, the five TeNT concentrations and the blanks were analysed in each assay for the test samples (TeNT in toxoid) and for the positive controls (TeNT in binding buffer) using a four-parameter logistic (4PL) regression model. Based on the resulting dose-response curves, the median effective dose ($ED_{50}$) values were calculated for both types of samples. The RP of the TeNT diluted in toxoid compared to TeNT diluted in buffer was then estimated by dividing the $ED_{50}$ value obtained for the test samples by the $ED_{50}$ value of the positive controls.

A one-way random analysis of variance (ANOVA) was performed to estimate the repeatability (i.e. variability between assays within a laboratory, pooled across the different laboratories) as well as the reproducibility (sum of the between-assay and between-laboratory variations) of the BINACLE. Both were expressed as % GCV of the RP estimates.

The LOD was estimated using a cut-off-based method [16]. The cut-off was calculated for each assay according to the formula:

$$Cut\text{-}off = Mean_{BLK} + 3.3 \times SD_{BLK}$$

where $Mean_{BLK}$ was the mean of the AU of all blank wells of a plate and the $SD_{BLK}$ was the corresponding standard deviation. The LOD of an assay was defined as the lowest TeNT concentration at which at least 80 % of absorbance values were above the cut-off value. The LOD per laboratory was defined as the lowest TeNT concentration at which at least 80 % of absorption values were above cut-off in three out of four assays. For the two laboratories that contributed only three valid assays, the LOD was the TeNT concentration at which at least 80 % of absorption values of all valid assays were above cut-off; for the laboratory that contributed six independent assays, 80 % of absorption values had to be above the cut-off in five out of six assays.

Descriptive plots, summary statistics and analyses of variance were generated using R statistical software version 4.1.1. The fitting of the 4PL regression model and the $ED_{50}$ calculation were carried out using CombiStats™ 7.0 [17, 18].

## 4. Results of the collaborative study

The experimental phase of the collaborative study took place between 8 February and 8 June 2023. Each of the nine study participants provided results of four BINACLE assays as requested in the study protocol. One of these participants carried out two additional assays over the course of the experimental phase so that, in total, the results of 38 assays were submitted.

Two different washing procedures were used by the participants: Four of the participants (Laboratories 2, 4, 6, 8) performed the washing steps manually while five (Laboratories 1, 3, 5, 7, 9) used an automated microplate washer. None of the participants changed the washing procedure between assays.

## 4.1. Documented protocol deviations

Laboratories 5, 6 and 7 reported no protocol deviations, while deviations were reported by Laboratories 1, 2, 3, 4, 8 and 9.

As requested, all participants read the absorption at 450 nm; 620 nm was used as reference wavelength by seven participants, and two used a 630 nm filter (Laboratories 2 and 4).

The shaking speed indicated in the study protocol (i.e. 250 rpm) was the shaking speed determined for the shaker model used in the laboratory of the project leader. Due to the specific characteristics of the devices used in their laboratories, three participants reported deviations regarding the shaking speed: Laboratory 1 carried out all 37 °C incubation steps at 100 rpm; the same steps were carried out at 400 rpm by Laboratory 3 and at 240 rpm by Laboratory 9.

Two participants reported deviations regarding the TeNT solution centrifugation: Laboratory 1 reported centrifugation at 90 x *g*, Laboratory 9 reported centrifugation at 80 x *g* in Assay 1 and at 90 x *g* in Assays 2 to 4 instead of 84 x *g* as specified in the protocol.

In addition, Laboratory 2 reported to have accidentally used PIPES buffer instead of PIPES/NaCl buffer for the preparation of the Binding Buffer in Assay 1 and possibly in Assay 4. In addition, in Assay 1, the plate was exposed to light for an unknown amount of time before reading the colorimetric signal and, in Assay 2, the volume of $H_2SO_4$ was doubled.

Laboratory 3 reported that the stock solutions of rSyb and tris(2-carboxyethyl)phosphine hydrochloride (TCEP) were vortexed in Assay 1.

Laboratory 8 accidentally switched off the heated plate shaker during the cleavage step in Assay 1.

## 4.2. Excluded values and assays

The majority of protocol deviations were judged to have had only a minor effect on the assay results, as analysis of the dose-response curves showed that the acceptance criteria were fulfilled despite the deviations. For example, Assays 1 and 4 reported by Laboratory 2 fulfilled the acceptance criteria and were therefore not excluded from the analysis, despite the use of the wrong buffer. However, the data from two assays (one assay performed by Laboratory 3 and one assay performed by Laboratory 8) as well as some individual values from various assays were excluded from further analysis for the reasons described in Table 1. Results of these assays are shown in the tables and figures of this article for information, but are not included in statistical analyses.

Table 1 – *Excluded assays and values*

| Participant | Assay / Well | Reason |
|---|---|---|
| Lab 3 | Assay 3 | Acceptance criteria not fulfilled, significant deviation from results of assays 1, 2 & 4 (cf. Figure 1) |
| Lab 8 | Assay 1 | Cleavage of synaptobrevin performed at room temperature |
| Lab 2 | Assay 2 / well A12 | Negative control: 1.196 AU at 450 nm (outlier) |
| Lab 4 | Assay 2 / well E2 | 3 ng/mL TeNT: 2.745 AU at 450 nm (outlier) |
| Lab 5 | Assay 3 / wells A4 - A7<br>Assay 4 / wells A3 - A7 | 9 ng/mL TeNT: AU > 4.00 at 450 nm (detector saturation) |
| Lab 7 | Assay 1 / well F9 | 0.33 ng/mL TeNT: 1.945 AU at 450 nm (outlier) |
| Lab 8 | Assay 2 / well G3 | 1 ng/mL TeNT: 2.034 AU at 450 nm (outlier) |
| Lab 9 | Assay 3 / well D3<br>Assay 1 / well E1<br>Assay 3 / well F1 | 1 ng/mL TeNT: 2.235 AU at 450 nm (outlier)<br>9 ng/mL TeNT: 1.778 AU at 620 nm (outlier)<br>9 ng/mL TeNT: 0.173 AU at 620 nm (outlier) |

## 4.3. Results of the negative and blank controls

Each participant provided six absorbance values per assay and blank condition, which was either toxoid or binding buffer without added TeNT incubated in wells containing ganglioside GT1b. The median absorbance values of the blank controls are shown in Table 2. There was only a limited difference between the medians obtained for binding buffer or for toxoid without added TeNT, indicating that the toxoid did not increase the background signal of the assay.

Similarly, in each assay, two replicates of each TeNT dilution were incubated in wells devoid of the TeNT receptor ganglioside GT1b (see plate layout in Annex II), resulting in 12 negative control wells for TeNT in binding buffer and 12 negative control wells for TeNT in toxoid per assay plate. For these control wells there was also only a limited difference between the solutions with and without toxoid (Table 2).

Within each condition, the absorbance values of the negative control wells containing 9 ng/mL TeNT were only marginally higher than those of the wells containing lower concentration samples, indicating that unspecific binding to the microplate in the absence of the TeNT receptor occurred only to a very small extent. The average difference of the negative control values between 0 ng/mL and 9 ng/mL TeNT was 0.044 AU for TeNT diluted in binding buffer and 0.059 AU for TeNT diluted in toxoid solution.

Overall, the results for the negative controls obtained by each participant were similar to those obtained for the blank controls: the individual absorbance values reported for the blank controls ranged from 0.08 to 0.45 AU, and for the negative controls from 0.09 AU to 0.49 AU.

Table 2 – *Median absorbance values and 95 % interval of the negative and the blank controls*

| | Laboratory | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Blank Control** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** |
| N | 24 | 24 | 18# | 24 | 24 | 24 | 36* | 18# | 24 |
| Buffer | 0.38 | 0.12 | 0.12 | 0.12 | 0.29 | 0.22 | 0.28 | 0.19 | 0.21 |
| Toxoid | 0.38 | 0.11 | 0.12 | 0.12 | 0.28 | 0.22 | 0.29 | 0.20 | 0.19 |
| I95 (pooled) | 0.17 | 0.13 | 0.05 | 0.07 | 0.18 | 0.08 | 0.06 | 0.19 | 0.10 |
| **Neg. Control** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** |
| N | 48 | 48$ | 36# | 48 | 48 | 48 | 72* | 36# | 48 |
| Buffer | 0.38 | 0.14 | 0.12 | 0.13 | 0.30 | 0.24 | 0.31 | 0.19 | 0.20 |
| Toxoid | 0.40 | 0.13 | 0.13 | 0.13 | 0.29 | 0.22 | 0.31 | 0.24 | 0.19 |
| I95 (pooled) | 0.19 | 0.15 | 0.10 | 0.09 | 0.26 | 0.08 | 0.16 | 0.21 | 0.13 |

N = number of observations; $ one atypical value excluded for the toxoid condition for which n = 47, # Laboratory 3, Assay 2 and Laboratory 8, Assay 1 were excluded; * Laboratory 7 performed 6 assays. I95 (pooled) = interval comprising 95 % of all values around the median of the pooled data.

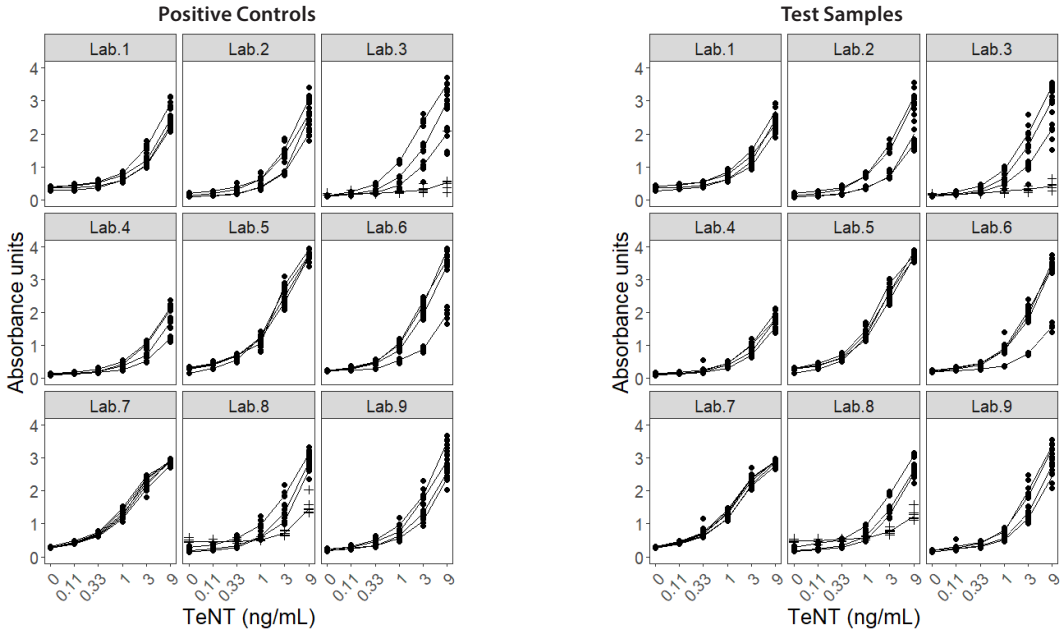
For both types of controls, a good correlation between results obtained for the two different conditions (binding buffer or toxoid) could be observed so that the data was pooled for the description of the distribution (Table 2).

## 4.4. Results of the positive controls and the test samples

TeNT diluted in binding buffer was the positive control in the assay. In total, six wells of each of the five different TeNT concentrations (0.11, 0.33, 1, 3, 9 ng/mL) and the blanks (0 ng/mL TeNT) were analysed per plate. Analysis of the dose-response curves showed that, by and large, the variability between the replicate wells on a single plate was small and the assays showed a clear, positive dose-response relationship. An exception to this were the data obtained by Laboratory 3 in Assay 2 and by Laboratory 8 in Assay 1, which were excluded from further analysis (Figure 1, data points marked by a plus sign).

With the exception of Laboratories 3 and 6, the curves of all assays obtained by a single participant for the positive controls were very similar; however, some differences between laboratories were observed (Figure 1, left). The dose-response curves obtained based on the data of Laboratory 7 were sigmoidal and included the start of the upper asymptote, whereas the upper asymptote was not included in the dose-response curves of the other participants. Laboratory 5 obtained particularly steep dose-response curves, with maximum values for the highest TeNT concentration above 4.0 AU, while Laboratory 4 obtained a comparatively low signal, approximately 2.0 AU, at the highest TeNT concentration.

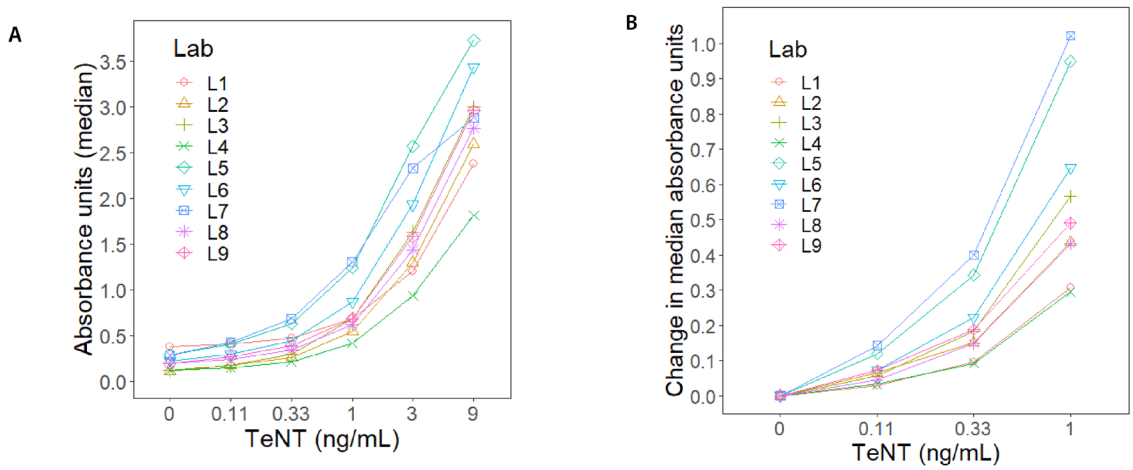
Figure 1 – *Dose-response curves for the individual assays carried out by each laboratory*




The assay results obtained for the positive controls (TeNT diluted in binding buffer) are shown on the left, the results obtained for the test samples (TeNT diluted in toxoid) are shown on the right. Each circle represents the measurement of one well; plus signs indicate the excluded assays (Laboratory 3 Assay 2 and Laboratory 8 Assay 1). The lines connect the median values of each assay.

The results obtained for the test samples containing TeNT diluted in toxoid Figure 1, right) were similar to the results obtained for the positive controls. In line with this observation, the calculated median absorbance values per laboratory for the positive controls and the test samples did not differ significantly, corroborating that the toxoid used in the study had no impact on the detection of TeNT by the BINACLE method (Annex IV, Table 1).

Figure 2 – *Median dose-response curves per laboratory*




A) Comparison of the median dose-response curves (pooled data of positive controls and test samples per laboratory) shows qualitatively similar responses between laboratories.
B) Normalised median dose-response curves up to 1 ng/mL TeNT (pooled data of positive controls and test samples). The results of the blank samples were set to 0 to illustrate the increase in absorbance units with increasing TeNT concentrations. At 0.11 ng/mL TeNT the median increase was between 0.028 and 0.143 AU.

As illustrated by the median dose-response curves shown in Figure 2 A, the per-laboratory results of the BINACLE assay fell into several groups. As the dose-response curve of Laboratory 7 reached a plateau at 9 ng/mL TeNT, the comparison between laboratories was mostly focused on the lower concentrations. At 3 ng/mL TeNT Laboratories 5 and 7 attained the highest signals, whereas Laboratory 4 obtained the lowest signals. The other participants formed an intermediate group with the exception of Laboratory 1 who obtained higher background signals. Normalisation of the data, to remove the baseline difference at 0 ng/mL TeNT, allowed a better comparison of the slopes of the dose-response curves (Figure 2 B), and revealed that the grouping of the laboratories remained largely the same. However, after baseline correction, the profile of Laboratory 1 became virtually identical to the profile of Laboratory 4 in the lower TeNT concentration range (Figure 2 B). For the purposes of Figure 2, the data for the positive controls and the test samples were pooled as there was only a very limited difference between the two sample types.

## 4.5. Assay repeatability and reproducibility

The RP of the samples containing TeNT in toxoid solutions relative to TeNT in binding buffer was calculated for each assay. Assay repeatability (within-laboratory variation) and reproducibility (within- and between-laboratory variation) were then assessed based on the resulting RP values. Consistent with the observation that the median absorbance values of the positive controls did not differ from those of the test samples, the geometric means (GM) of the RP estimates per laboratory ranged between 0.93 and 1.22 for the individual participants (Figure 3 and Annex IV, Table 2) with a grand mean of 1.03.

The within-laboratory variability of the RP results ranged from 3 % to 20 % GCV for the individual participants and was found to be equal to 12 % GCV on average. The between-laboratory variability was 4 % GCV and did not contribute much to the assay reproducibility, which was 13 % GCV when calculated for a single assay and was thus only marginally higher than the within-laboratory variability alone (Table 3). If needed, the reproducibility could be improved by choosing a test format consisting of multiple runs: if, for example, combined data from three BINACLE runs are used for the calculations, the standard error about the reportable value would then be 8.2 % GCV.

Figure 3 – *Frequency distribution of the RP estimates*

Each square represents the RP of one assay, the number in the square and its colour indicate the respective participant. All 36 calculated RP values were in the range of 0.72 to 1.38; the distribution was centred on 1.0.

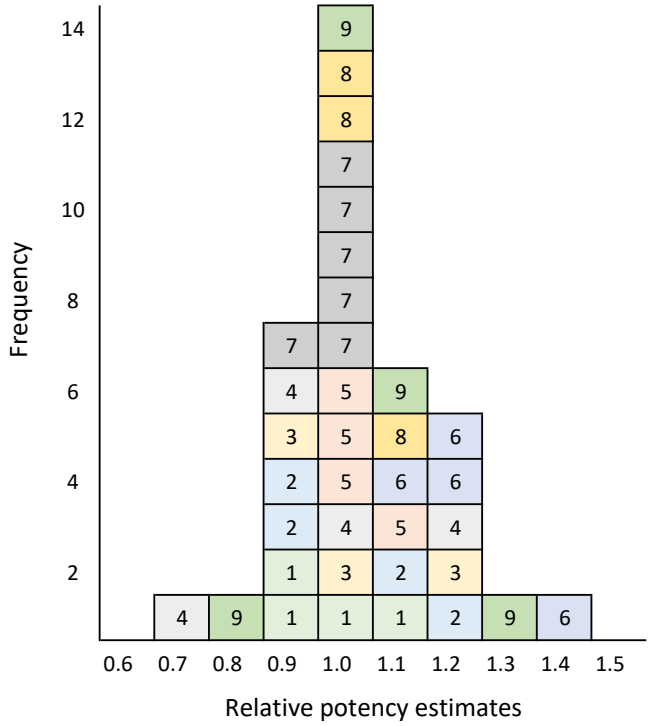


Table 3 – *Assay variability and reproducibility*

| Variability [GCV] | Laboratory | | | | | | | | | average |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | |
| Intra-laboratory | 9 % | 16 % | 12 % | 20 % | 3 % | 10 % | 5 % | 5 % | 19 % | 12 % |
| Inter-laboratory | 4 % | | | | | | | | | |
| Reproducibility (1 assay result) | 13 % | | | | | | | | | |

## 4.6. Limit of detection (LOD)

The applicability of the BINACLE assay for the testing of tetanus toxicity in toxoids will depend on its ability to detect insufficiently detoxified antigen bulks that are also detected by the current *in vivo* test, an ability which is linked to the LOD of each of the two methods. One important factor influencing the LOD is the signal increase induced by the TeNT spikes compared to the blank controls. This average increase per laboratory is illustrated in Figure 2 B.

Table 4 – *Percentage of absorbance values above the assay cut-off*

| Lab | Assay | TeNT in Binding Buffer [ng/mL] | | | | | TeNT in Toxoid [ng/mL] | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 9 | 3 | 1 | 0.33 | 0.11 | 9 | 3 | 1 | 0.33 | 0.11 |
| 1 | 1 | 100 | 100 | 100 | 100 | 0 | 100 | 100 | 100 | 100 | 33 |
| | 2 | 100 | 100 | 100 | 100 | 83 | 100 | 100 | 100 | 100 | 100 |
| | 3 | 100 | 100 | 100 | 100 | 0 | 100 | 100 | 100 | 100 | 0 |
| | 4 | 100 | 100 | 100 | 33 | 0 | 100 | 100 | 100 | 67 | 0 |
| 2 | 1 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| | 2 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| | 3 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| | 4 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| 3 | 1 | 100 | 100 | 100 | 100 | 67 | 100 | 100 | 100 | 100 | 50 |
| | *2 (excl.)* | *83* | *83* | *67* | *0* | *17* | *100* | *83* | *83* | *0* | 0 |
| | 3 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| | 4 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| 4 | 1 | 100 | 100 | 100 | 100 | 33 | 100 | 100 | 100 | 100 | 100 |
| | 2 | 100 | 100 | 100 | 100 | 0 | 100 | 100 | 100 | 100 | 0 |
| | 3 | 100 | 100 | 100 | 100 | 67 | 100 | 100 | 100 | 100 | 100 |
| | 4 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| 5 | 1 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| | 2 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| | 3 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| | 4 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| 6 | 1 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| | 2 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| | 3 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| | 4 | 100 | 100 | 100 | 100 | 17 | 100 | 100 | 100 | 83 | 0 |
| 7 | 1 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| | 2 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| | 3 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| | 4 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| | 5 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| | 6 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| 8 | *1 (excl.)* | *100* | *100* | *0* | *0* | *0* | *100* | *100* | *17* | *0* | 0 |
| | 2 | 100 | 100 | 100 | 100 | 17 | 100 | 100 | 100 | 100 | 17 |
| | 3 | 100 | 100 | 100 | 100 | 17 | 100 | 100 | 100 | 100 | 17 |
| | 4 | 100 | 100 | 100 | 100 | 33 | 100 | 100 | 100 | 100 | 83 |
| 9 | 1 | 100 | 100 | 100 | 100 | 50 | 100 | 100 | 100 | 83 | 0 |
| | 2 | 100 | 100 | 100 | 100 | 0 | 100 | 100 | 100 | 100 | 0 |
| | 3 | 100 | 100 | 100 | 100 | 50 | 100 | 100 | 100 | 100 | 50 |
| | 4 | 100 | 100 | 100 | 100 | 83 | 100 | 100 | 100 | 100 | 83 |
| **% assays above cut-off** | | **100** | **100** | **100** | **97** | **61** | **100** | **100** | **100** | **97** | **69** |

To formally assess the LOD of the BINACLE assay, a cut-off-based approach was chosen and the analysis was carried out following the same principles that were applied to the data obtained in the first collaborative study. Cut-off values were calculated for each assay (Annex IV, Table 3). For each tested TeNT concentration, the percentage of wells with absorbance values above the assay cut-off was calculated (Table 4). In the 36 valid assays, TeNT was detected at a concentration of 0.11 ng/mL in 22 assays (61 %) when diluted in binding buffer (positive controls) and in 25 assays (69 %) when diluted in toxoid (test samples); 0.33 ng/mL TeNT was reliably detected in 97 % of the assays (35 out 36), irrespective of the dilution in buffer or in toxoid. At higher TeNT concentrations, the toxin detection rate was 100 %.

Analysis of the data at the level of the individual participants showed that all participants detected 0.11 ng/mL TeNT diluted in toxoid in at least one of their assays. For three participants (33 %, Laboratories 2, 5, 7), the LOD was 0.11 ng/mL in all assays. The overall LOD per participant was 0.11 ng/mL TeNT in toxoid for five out of nine laboratories (Laboratories 2, 4, 5, 6 and 7) and 0.33 ng/mL for four out of nine laboratories (Laboratories 1, 3, 8 and 9).

## 5. Results of the additional tests using concentrated toxoid

To assess the dose-response curves and the LOD of the BINACLE assay when concentrated toxoids are tested, additional assays were performed in the laboratory of the project leader using the toxoid and the TeNT used in the collaborative study. In a first step the AU recorded for the blank controls were analysed. The comparison indicated that increasing the toxoid concentration had only a small influence on the blank results (Table 5).

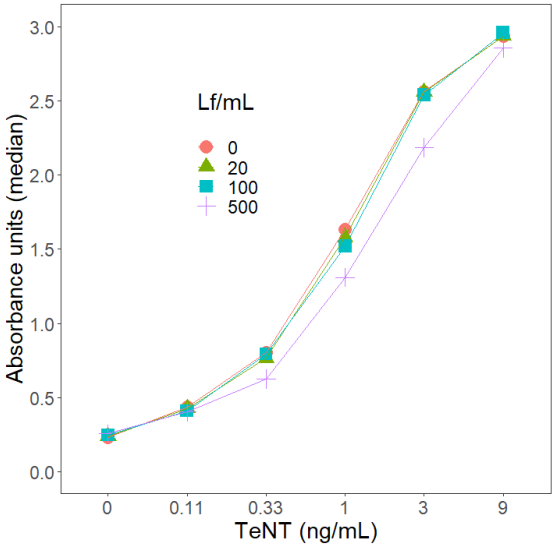
Table 5 – *Mean and SD of the blank controls and the resulting cut-off absorbance values per assay*

| | $Mean_{BLK}$ | | | $SD_{BLK}$ | | | Cut-off values | | |
|---|---|---|---|---|---|---|---|---|---|
| Toxoid concentration [Lf/mL] | Assay | | | Assay | | | Assay | | |
| | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| **0** | 0.235 | 0.243 | 0.255 | 0.017 | 0.015 | 0.002 | 0.291 | 0.294 | 0.261 |
| **20** | 0.240 | 0.252 | 0.270 | 0.024 | 0.021 | 0.006 | 0.320 | 0.322 | 0.289 |
| **100** | 0.253 | 0.267 | 0.284 | 0.026 | 0.020 | 0.003 | 0.338 | 0.332 | 0.293 |
| **500** | 0.266 | 0.280 | 0.305 | 0.030 | 0.029 | 0.008 | 0.367 | 0.376 | 0.331 |

Comparison of the dose-response curves generated for the TeNT spikes diluted in 0, 20, 100 and 500 Lf/mL showed a strong overlap up to 100 Lf/mL of toxoid. However, slightly lower absorption values were obtained when the toxin was diluted in 500 Lf/mL of toxoid (Figure 4), possibly due to competition between toxoid and TeNT molecules for the receptor binding sites.

Figure 4 – *Median dose-response curves of TeNT diluted in toxoid*

Each curve represents the results obtained for the dilutions of the different TeNT spikes (0, 0.11, 0.33, 1, 3 and 9 ng/mL) in one toxoid concentration as indicated in the figure legend.
n = 3 assays.



As for the collaborative study, cut-off absorbance values were calculated as a basis for the estimation of the LOD (Table 5). Table 6 shows the percentage of absorbance values above the cut-off value for any given combination of toxoid and toxin concentration; 0.11 ng/mL of TeNT could be successfully detected in all assays up to 100 Lf/mL of toxoid and in two out of three assays when diluted in 500 Lf/mL of toxoid. This result suggests that the LOD was only minimally impacted by the increasing toxoid concentration.

Table 6 – *Percentage of absorbance values above cut-off*

| Toxoid [Lf/mL] | | 0 | | | 20 | | | 100 | | | 500 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | A1 | A2 | A3 | A1 | A2 | A3 | A1 | A2 | A3 | A1 | A2 | A3 |
| TeNT [ng/mL] | 9.0 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| | 3.0 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| | 1.0 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| | 0.33 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| | 0.11 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 0 | 100 | 100 |

## 6. Discussion

The second collaborative study phase of BSP136 was initiated to test whether increased method standardisation alongside protocol optimisation and simplification would improve repeatability and reproducibility of the BINACLE assay compared to the results obtained in the first collaborative study [15]. The LOD of the method is a crucial indicator for the applicability of the BINACLE assay as a potential alternative to the *in vivo* test for *Absence of tetanus toxin* (Ph. Eur. monographs *0452* and *0697* [1, 2] and was to be assessed under the optimised conditions. As part of the optimisation, the vast majority of reagents were pretested in the laboratory of the project leader and provided to the study participants in a ready-to-use format. Additionally, critical steps in the SOP were modified, e.g. to prevent drying of the wells during pipetting steps. To reduce method complexity, only one toxoid was included in the second collaborative study on one microplate per assay run, while in the first collaborative study four toxoids were analysed, which required the parallel handling of two microplates in each test [15]. The inclusion of only one toxoid allowed the collection of data for five different TeNT spike concentrations, 0.11, 0.33, 1.0, 3.0 and 9.0 ng/mL TeNT, compared to the first study where only three different TeNT concentrations were analysed. These TeNT spike concentrations were chosen to cover the estimated LOD of the *in vivo* test and to include the upper and lower asymptote of the dose-response curve in case of optimal assay performance. In comparison to the first collaborative study, in which 0.1 ng/mL, 0.5 ng/mL and 5 ng/mL TeNT spikes were tested, the higher number of TeNT spikes in the low concentration range, which were included in the study reported here, allowed a more detailed evaluation within the expected range of the method's LOD.

The concentration of the toxoid that was chosen as a matrix for TeNT was 20 Lf/mL. This is the toxoid concentration of some relevant tetanus vaccines for human use and was selected because of the requirement to test "the same concentration as in the final vaccine" in the irreversibility arm of the compendial test *Absence of tetanus toxin and irreversibility of toxoid* which was prescribed in the Ph. Eur. *0452* version that was valid at the time the first collaborative study of BSP136 was designed. Even though the test for irreversibility has since been removed, and the test *Absence of tetanus toxin* prescribes the testing of 500 Lf/mL toxoid, the lower toxoid concentration was also used in the second collaborative study as a matrix for TeNT for better comparability to the earlier results. In additional tests carried out in the context of the present collaborative study, the project leader assessed the LOD when 500 Lf/mL of the study toxoid was analysed and observed only a very limited effect of the higher toxoid concentration on the LOD.

Overall, the participants of the current study carried out the BINACLE assay following the provided SOP without difficulties, and all obtained good dose-response curves for the TeNT-spiked samples. The curves obtained by all laboratories displayed strong positive relationships between the toxin concentrations and the resulting assay signals, whereas in the first collaborative study, only seven out of the 19 participants could obtain signals that showed a strong dose-response relationship [15]. This improvement is an indicator of the positive impact of the implemented method changes on the performance characteristics.

Repeatability and reproducibility of the method were assessed based on the variability of the RP. The resulting repeatability ranged from 3 % to 20 % per laboratory and was on average 12 % GCV. The reproducibility of the method was 13 % GCV. These data indicate that the method's precision is in the range commonly reported for immunochemical assays and highlight the reliability of the BINACLE method when carried out under optimised conditions.

The LOD was determined for each assay using a cut-off-based method [16]. Using this approach, the LOD was 0.11 ng/mL TeNT, the lowest TeNT spike concentration included in the second collaborative study, in at least one assay for each laboratory, and in all assays for three participants. Given the reliable detection of 0.11 ng/mL TeNT for those three participants, it is probable that a lower estimate of the LOD would have been found, had lower concentrations been included in the study design; 0.33 ng/mL TeNT was successfully detected in 97 % of all valid assays. The LOD per laboratory was 0.11 ng/mL for five out of nine participants (56 %) and 0.33 ng/mL for four out of nine participants (44 %). This was a clear improvement compared to the earlier collaborative study, where the LOD had been above 0.5 ng/mL for 63 % of the participants, and above 5 ng/mL TeNT for 21 % of the participants. However, also in this first collaborative study, some of the participants had obtained LODs close to 0.1 ng/mL [15].

Any *in vitro* method that is to be considered as an alternative to an existing safety test for specific toxicity should, according to Ph. Eur. general text *5.2.14*, be at least as sensitive as the existing *in vivo* method. For this comparison, historic *in vivo* data can be used [19]. The analytical method parameter to be compared for this purpose is the LOD of the two detection methods. However, in the present case, publicly available data regarding the LOD of guinea pigs for TeNT is scant and variable. In a classical review article, the lethal dose for guinea pigs has been extrapolated from historic data to be 0.3 ng/kg [3], whereas direct experimental evidence generated by a European vaccines manufacturer and following the method described in Ph. Eur. monograph *0452* suggested that guinea pigs were sensitive to TeNT at a concentration of 1.5 ng/kg [4]. The minimum five-fold difference in the apparent sensitivity of guinea pigs to TeNT reported in those two publications can be attributed partially to differences in the animal strains and in the purity and specific activity of the toxin preparations, to the presence or absence of toxoids and to the use of unclear conversion factors in one of the reports [3]. This difference illustrates the difficulty of assigning a concrete detection limit to the *in vivo* test based on data derived from different sources and highlights the fact that, while such historically validated animal assays have served their purpose, they were developed before the current standards of validation (e.g. ICH Q2 [20]) were in place.

To respect the 3R principles, and for scientific reasons, such as the lack of standardisation of the animal test, the example TeNT/toxoid combination used for the present study was not tested *in vivo*. Thus, a direct comparison of the *in vitro* and *in vivo* LOD was not in the scope of BSP136. However, to obtain a general idea, the available published data on the *in vivo* LOD can be used as an approximation, keeping the caveats associated with these values in mind. From a conceptual perspective, the unit of detection in the *in vivo* test is one guinea pig, while in the *in vitro* test the unit of detection would be one well in a microplate. For easier comparison, absolute doses of TeNT can be used. Assuming the LOD of the animal model to be 1.5 ng/kg [4] and using the animal weight range of 250 to 350 g described in Ph. Eur. monographs *0452* and *0697*, the minimal dose that could be detected by injection of a TeNT solution into a guinea pig would be 0.375 ng TeNT when using animals weighing 250 g, or 0.525 ng TeNT when using animals weighing 350 g. If the same calculations were to be made using 0.3 ng/kg [3] as the LOD for guinea pigs, the detectable TeNT doses for animals weighing 250 g or 350 g would be 0.075 ng and 0.105 ng, respectively. In the present study, TeNT could reliably be detected in the BINACLE assay at concentrations of 0.11 ng/mL and 0.33 ng/mL, which correspond to detectable doses of 0.011 ng and 0.033 ng TeNT as 0.1 mL of TeNT solution per well was tested.

Accordingly, the BINACLE assay is clearly more sensitive than the animal test with respect to the absolute toxin doses that can be detected: the BINACLE was capable of detecting between 0.011 ng and 0.033 ng TeNT, while the estimated LOD of the guinea pig lies between 0.075 ng and 0.525 ng TeNT.

As mentioned above, a key requirement for any replacement test is to reliably detect samples that do not comply with the compendial test. For the comparative assessment of the test for *Absence of tetanus toxin* [1, 2] using an *in vitro* readout (BINACLE) and an *in vivo* readout (guinea pig), additional factors besides the LOD, such as the toxoid concentration and the testable volume, need to be considered. Using the parameters of the compendial test (1 mL of 500 Lf/mL of toxoid solution injected per animal, guinea pig weight range 250 – 350 g) in combination with the publicly available data on the *in vivo* sensitivity as discussed above, the minimum toxin concentration in toxoid solutions that can be expected to elicit tetanus symptoms in the animal test is approximately in the range between 0.075 and 0.525 ng/mL. Based on the outcome of the study

presented here, the lowest TeNT concentration in toxoid solutions that can be identified by the BINACLE method is presumably between 0.11 to 0.33 ng/mL. The additional experiments carried out in the laboratory of the project leader showed that for a suitable toxoid, sensitive toxin detection by the BINACLE assay can not only be achieved in diluted toxoid solutions of 20 Lf/mL as used in the collaborative studies, but may also be achieved in concentrated toxoid solutions of 500 Lf/mL as currently prescribed for the animal test. Taking this finding into account, the comparison suggests that for toxoids suitable for testing at the same concentration *in vitro* and *in vivo*, the pass/fail decision would be the same for the two methods.

However, due to the impact of various parameters, such as the purity and specific activity of the respective TeNT batch, the characteristics of the toxoid and the non-standardisation of the *in vivo* test on the sensitivities of the respective methods, an individual, laboratory- and product-specific comparative assessment should be made during method validation.

To achieve successful implementation of the method, the following points should be observed:

A) Establishing suitability for specific toxoids

It has been shown that toxoids from some sources induce high signals in the BINACLE assay [13]. It is therefore recommended that users establish the suitability of the method for the testing of their specific toxoid by elaborating dose-response curves for several toxoid batches that have passed the *in vivo* test *Absence of tetanus toxin* according to Ph. Eur. monographs *0452* or *0697* [1, 2]. Specifically, it should be evaluated if the toxoid, when tested in the absence of active toxin, elicits any elevated background signals, or if it exerts any other effects that might impede sensitive toxin detection, especially when used at higher concentrations (e.g. 500 Lf/mL). In addition, it should be examined if the BINACLE signal varies across different batches of toxoid.

B) LOD and comparison of sensitivity

Using an in-house reference TeNT, the LOD of the BINACLE method should be determined by the user following a cut-off-based method [16] and compared to the sensitivity of the currently used *in vivo* test for *Absence of tetanus toxin*. Spiked toxoid solutions that mimic non-compliant toxoid batches should be included in these studies to ensure that all toxin-containing sample solutions detected by the *in vivo* method are also reliably detected in the BINACLE assay. Any differences between the two assays with respect to tested toxoid concentration and sample volume need to be considered during method validation in order to ensure continued safety of the vaccines.

C) Routine use

To ensure adequate sensitivity of the assay in routine use, control wells containing an in-house reference toxoid that is representative of the vaccine antigen should be included on each assay plate. This reference toxoid should be included in plain form as well as spiked with a reference TeNT at concentrations covering the LOD. The test can be considered valid if TeNT is detected at the expected concentration. The tested toxoid would comply with the test if the AU measured for the test toxoid alone does not exceed the cut-off determined for the in-house reference toxoid of the same concentration without added TeNT in the same assay.

D) Critical reagents

Critical reagents for the BINACLE assay that were contract-manufactured according to protocols developed by the institution of the project leader are the rSyb and the cleavage-site-specific anti-synaptobrevin antibody. Both length and correct folding of the rSyb determine its suitability for use in the BINACLE assay and for each batch efficient cleavage by TeNT should be confirmed. Further, the specificity of the anti-synaptobrevin antibody for the cleaved synaptobrevin-2 fragment needs to be demonstrated. An example production and characterisation strategy for both has been published [21] and a more detailed production protocol for rSyb is provided in Annex II.

Due to the lack of an official TeNT reference toxin, users should establish an in-house reference TeNT simultaneously to the comparison of the sensitivity of the *in vivo* and *in vitro* tests. Any new batch of in-house reference TeNT should then be suitably qualified in the BINACLE assay to ensure that the specific activity of the reference toxin and its performance in the BINACLE test are consistent with the previous batch.

In addition, an in-house reference toxoid should be established that is representative of the vaccine antigen and that fulfils the requirements described above.

New batches of all reagents should be used only after careful prequalification.

## 7. Conclusions

The BINACLE, as a functional *in vitro* assay, reproduces the two most important specific steps of the mechanism of action of TeNT, i.e. the receptor binding and the subsequent proteolytic cleavage of the target protein. The study results demonstrate that the protocol changes introduced in the second collaborative study, together with the standardisation of the reagents, led to assay repeatability and reproducibility in the commonly reported range for immunochemical assays and to an LOD for TeNT in the estimated range of the *in vivo* LOD. This implies that compliance with a defined and proven SOP and the careful prequalification of reagent batches are prerequisites for achieving sensitive detection of TeNT in the BINACLE assay. However, while sensitive detection of TeNT has been shown to be possible in several toxoids in multiple studies and in the BSP136 project, considering the body of data that has been collected for the BINACLE, the applicability of the method for the testing of toxoids of different origins will need to be established in a product-specific manner. Additionally, due to the lack of data regarding the *in vivo* detection limit, each user needs to validate the BINACLE assay in comparison to the sensitivity of their current *in vivo* test and establish conditions for regular use.

Most importantly, the study results indicate that with appropriate in-house validation, the BINACLE assay could replace guinea pigs for the safety testing of various toxoids relevant for human and veterinary tetanus vaccines. Implementation of this scientifically relevant *in vitro* method would reduce the use of animals for the safety testing of vaccines in addition to reducing testing time and cost. Therefore, the BINACLE assay was proposed to Ph. Eur. expert Groups 15 and 15 V for consideration as an acceptable alternative to the current *in vivo* test *Absence of tetanus toxin* described in Ph. Eur. monographs *0452* and *0697*.

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## 9. List of participants (in alphabetical order by country)

J. MENON, Sanofi, Canada
E. COPPENS, Sanofi, France
S. SHAID, GSK Vaccines GmbH, Germany
H. BEHRENSDORF-NICOL, Paul-Ehrlich-Institut, Germany
B. SIKLODI, L. KISS, Ceva-Phylaxia Veterinary Biologicals Co. Ltd, Hungary
K. VERHEIJEN, L. LEVELS, Bilthoven Biologicals, Netherlands
C. FERNÁNDEZ, CZ Veterinaria, Spain
L. BARRIOLUENGO, Laboratorios Syva, Spain
S. FRAGOEIRO, MSD Animal Health, United Kingdom

## 10. Abbreviations

ANOVA: one-way random analysis of variance; 4PL: four-parameter logistic; AU: absorbance units; BB: Binding buffer; BLK: blank; BP: Binding plate; BSP: Biological Standardisation Programme; CP: Cleavage plate; $ED_{50}$: effective dose 50; EDQM: European Directorate for the Quality of Medicines & HealthCare; GCV: geometric coefficient of variation; GM: geometric mean; GT1b: ganglioside GT1b; IgG: immunoglobulin G; LOD: limit of detection; PEI: Paul-Ehrlich-Institut; Ph. Eur.: European Pharmacopoeia; RP: relative potency; rpm: revolutions per minute; rSyb: recombinant synaptobrevin; RT: room temperature; SD: standard deviation; SOP: Standard Operating Procedure; TCEP: tris(2-carboxyethyl)phosphine hydrochloride; TeNT: tetanus neurotoxin; TMAO: trimethylamine *N*-oxide dihydrate; TMB: 3,3',5,5'-tetramethylbenzidine; Txd: toxoid; WHO: World Health Organization.

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## Annexes

Annex I – Materials and equipment required to perform the BINACLE assay

Annex II – Supplemental method information: BINACLE assay

Annex III – Protocol for expression and purification of recombinant synaptobrevin-2 (rSyb)

Annex IV – Supplemental study results

## Annex I – Materials and equipment required to perform the BINACLE assay

Table 1 – *Materials and reagents provided to each participant*

| Label | Material/Reagent | Amount |
|---|---|---|
| **TeNT 1 – TeNT 6**<br>(store at -70 ± 10 °C) | **TeNT 2 × stock solution**<br>Sigma-Aldrich, T3194<br>TeNT diluted in 100 mM PIPES / 150 mM NaCl, pH 6.4 / 1 % BSA; blinded for the study.<br>TeNT 1 – 18 ng/mL; TeNT 2 – 6 ng/mL; TeNT 3 – 2 ng/mL, TeNT 4 – 0.67 ng/mL; TeNT 5 – 0.22 ng/mL, TeNT 6 – 0 ng/mL | 4 sets of<br>6 vials<br>(1 mL each) |
| **rSyb**<br>(store at -70 ± 10 °C) | **Recombinant synaptobrevin (rSyb)**<br>Contract-manufactured by toxologics GmbH,<br>Stock solution: 175 µM | 4 × 70 µL |
| **Biotin-conjugated Goat anti-Rabbit IgG**<br>(store at -25 ± 10 °C) | **Goat IgG anti-Rabbit IgG (H+L)-Biotin, MinX Hu,Ms,Rt**<br>Dianova, 111-065-144<br>Stock solution: ca. 0.6 - 0.7 mg/mL in Aqua dest./50 % glycerol | 1 × 80 µL |
| **GT1b**<br>(store at -25 ± 10 °C) | **Ganglioside GT1b**<br>Sigma-Aldrich, G3767<br>Stock solution: 1 mg/mL in methanol | 4 × 500 µL |
| **Peroxidase-conjugated streptavidin**<br>(store at -25 ± 10 °C) | **Streptavidin-HRPO**<br>Dianova, 016-030-084<br>Stock solution: 0.5 mg/mL in Aqua dest./50 % glycerol | 1 × 40 µL |
| **TCEP**<br>(store at -25 ± 10 °C) | **Tris(2-carboxyethyl)phosphine hydrochloride** Sigma-Aldrich, C4706<br>Stock solution: 500 mM | 4 × 100 µL |
| **anti-Syb Antibody**<br>(store at 5 ± 3 °C) | **Anti-Synaptobrevin Antibody**<br>Contract-manufactured by Biotrend, rabbit polyclonal<br>Stock solution: ca. 5 µg/mL in PBS/ 1 % BSA | 1 × 50 µL |
| **Asolectin**<br>(store at 5 ± 3 °C) | **Asolectin**<br>Sigma-Aldrich, 11145<br>Stock solution: 40 mg/mL in PBS | 1 × 1200 µL |
| **BSA**<br>(store at 5 ± 3 °C) | **Albumin bovine Fraction V, Protease-free**<br>Serva 11926; lyophilised | 1 × 8 g |
| **PIPES Buffer**<br>(store at 5 ± 3 °C) | **PIPES Buffer**<br>100 mM, pH 6.4 | 1 × 500 mL |
| **PIPES / NaCl Buffer**<br>(store at 5 ± 3 °C) | **PIPES / NaCl Buffer**<br>100 mM PIPES / 150 mM NaCl, pH 6.4 | 1 × 100 mL |
| **Tetanus Toxoid**<br>(store at 5 ± 3 °C) | **Tetanus Toxoid**<br>Routine production batch, compliant with test for *Absence of tetanus toxin*<br>Stock solution, 3413 Lf/mL | 1 × 500 µL |
| **TMB**<br>(store at 5 ± 3 °C) | **BioFX TMB Super Slow One Component HRP Microwell Substrate (Surmodics)**<br>Cliniscience, SU-TTMB-1000-01 | 1 × 60 mL |
| **PBS (10 ×)**<br>(store at RT) | **PBS solution (10 ×), without $Ca^{2+}$, without $Mg^{2+}$**<br>BIO&SELL, BS.L1835; 10 × stock solution | 3 × 500 mL |
| **TMAO**<br>(store at RT) | **Trimethylamine *N*-oxide dihydrate**<br>Sigma-Aldrich, 92277; powder | 1 × 16 g |
| **Tween 20**<br>(store at RT) | **Tween 20**<br>Sigma-Aldrich, P7949 | 1 × 10 mL |
| **Microplates** | **Multiwell immuno plate, MaxiSorp™**<br>Sigma-Aldrich, M5785 | 8 plates |

Table 2 – *Reagents to be provided by the participant*

| Reagent | Storage Temp. |
|---|---|
| **Aqua dest.** (sterile)<br>**Note:** Other water types of comparable quality (e.g. "high-purity water", "ultrapure water") can also be used. In any case, the water must be sterile. | RT |
| **Formaldehyde 8 %**<br>Prepare an **8 % formaldehyde solution** and fill the plastic container with screw cap (see section 2.2) to ½ with this solution. Label this container with a "toxic" symbol and put it into the safety cabinet where the assay will be performed. This container will be needed for the disposal of toxin-containing liquid waste. | RT |
| **HCl** (1 M) | RT |
| **Sulphuric acid, $H_2SO_4$** (1 M) | RT |

Table 3 – *Materials to be provided by the participant*

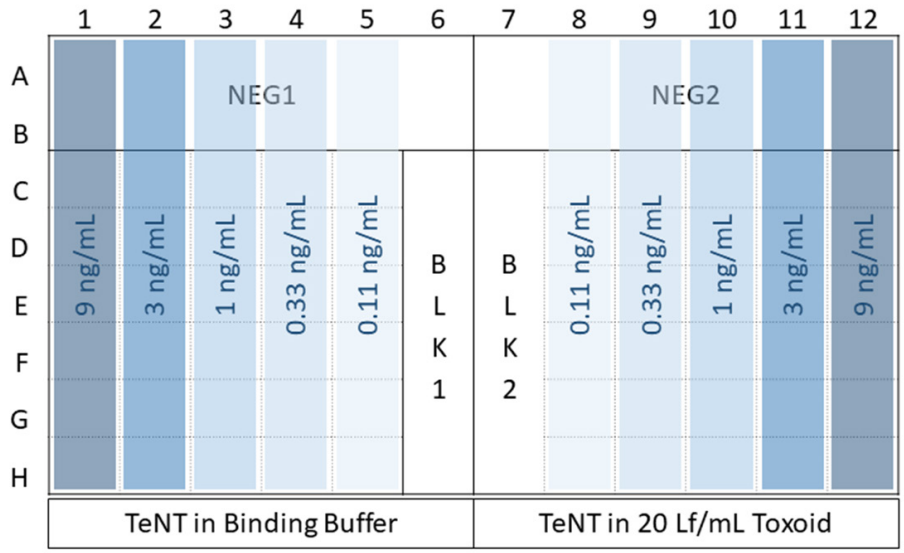
| Material |
|---|
| **Absorbent paper** |
| **Adhesive foils** (e.g. Greiner, order no.: 676001); approximately 40 sheets will be needed in total |
| **Combitips** (for use with Multipette; 2.5 mL, 5 mL) |
| **5-litre bottles** (or other suitable vessels) for preparation of PBS and Wash Buffer |
| **Plastic container** with screw cap for liquid toxin waste (ideally the opening of the container is wide enough to use with a multichannel pipette) |
| **Plastic reagent reservoirs** for use with multichannel pipettes |
| **PP-tubes** (15 mL, 50 mL) |
| **Serological pipettes** (5 mL, 10 mL, 25 mL) |
| **Sterile filters** 0.2 µm (syringe filters) |
| **Syringes** 20 mL, 50 mL |
| **Sterile pipette tips** (filter tips should be used for work with TeNT-containing solutions) |

Table 4 – *Instruments to be provided by the participant*

| Instruments |
|---|
| **Adjustable 12-channel pipettes** (300 µL, 250 µL, 100 µL, 50 µL) |
| **Adjustable volume pipettes** (10 µL, 100 µL, 1000 µL) with sterile (filter) tips |
| **Analytical balance** |
| **Centrifuge for 50 mL PP-tubes** |
| **Magnetic stirrer** |
| **Microbiological safety cabinet** |
| **Microplate shaker for incubation at RT** |
| **Microplate washer** (optional; alternatively, plates can be washed manually using a 12-channel pipette) |
| **Multipette for use with Combitips** |
| **Microplate photometer** (filters: 450 nm and 620 nm as reference wavelength) |
| **Microplate thermoshaker**, temperature-controlled, for incubation steps at 37 °C |
| **Pipetting aid** |
| **pH meter** |
| **Vortex shaker** |

## Annex II – Supplemental method information: BINACLE assay

### 1. Plate layout




The concentrations indicated in the plate layout refer to the TeNT concentration in the well.

NEG 1 and NEG 2: microwells without ganglioside GT1b

BLK 1 and BLK 2: 0 ng/mL TeNT

Positive controls: columns 1 - 5, Rows C – H

Test samples: columns 8 - 12, Rows C – H

### 2. Standard Operating Procedure – BINACLE assay

#### General notes:

- Use aseptic technique when opening bottles or tubes containing sterile solutions in order to keep the contents sterile.
- All microplate washing steps can either be performed using a 12-channel pipette or a microplate washer.
    - If using a microplate washer: set the soak time to 5 seconds unless otherwise indicated in the protocol, and aspirate after the final wash to remove all excess liquid.
    - If using a 12-channel pipette: when adding Wash Buffer to the plate, always start with row A and then proceed towards row H. After each wash, remove the Wash Buffer by inverting the plate and tapping it on absorbent paper. Rotate the plate and tap again on absorbent paper (to ensure uniform washing conditions for the wells on each side of the plate). After the final wash, make sure that all excess liquid is removed by tapping the plate on absorbent paper particularly thoroughly.

#### Day 0: Preparatory procedures

**Note: The buffers described in section 2.1. should be prepared freshly for each test (ideally, they should be prepared 1-2 days before the test is started).**

## 2.1. Preparation of PBS (1×) and Wash Buffer

### 2.1.1. PBS (1×)

- Add 350 mL PBS (10×) to 3150 mL aqua dest. and mix using a magnetic stirrer.
- Adjust pH to 7.1 using 1 M HCl.
- Transfer 500 mL of the PBS (1×) into a fresh bottle.

### 2.1.2. Wash Buffer (PBS / 0.05 % Tween 20)

- To the remaining 3000 mL PBS (1×) (from step 2.1.1.), add 1.5 mL Tween 20 (use of Combitip is recommended) and mix using a magnetic stirrer.

**Store PBS (1×) and Wash Buffer at RT during the whole assay.**

## Day 1: Preparation of microplates and binding step

## 2.2. Preparation of fresh buffers

### 2.2.1. Blocking Buffer (PBS / 1 % BSA / 100 µg/mL asolectin)

- Prepare Blocking Buffer by combining:

  60 mL PBS (1×) (from step 2.1.1)

  + 0.6 g BSA (lyophilised)

- Dissolve using a magnetic stirrer for approx. 30 minutes.
- Add 150 µL of asolectin stock solution. Transfer the remaining asolectin stock solution to the refrigerator (5 ± 3 °C) (it will be needed again on day 2 and for tests 2-4).
- Dissolve using a magnetic stirrer, filtrate using a syringe filter (0.2 µm), and store at RT until use.

### 2.2.2. Binding Buffer (100 mM PIPES / 150 mM NaCl, pH 6.4 / 1 % BSA)

- Prepare Binding Buffer by combining:

  20 mL PIPES / NaCl Buffer

  + 0.2 g BSA (lyophilised)

  Dissolve using a magnetic stirrer, filtrate using a syringe filter (0.2 µm), and store at RT until use.

## 2.3. Preparation of the microplate for TeNT binding

### 2.3.1. Coating the binding plate with ganglioside GT1b

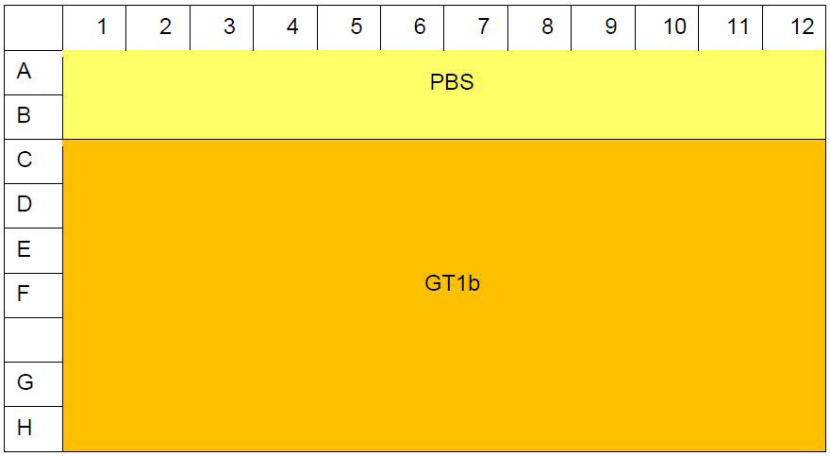
- Preheat a microplate thermoshaker to 37 °C.
- Take one vial of the GT1b stock solution out of the freezer and allow it to equilibrate to RT.
- Take two 15 mL PP-tubes and label one tube with “GT1b” and the other tube with “PBS”.
- Add 7.7 mL PBS (1×) to each of these tubes.
- Mix the GT1b stock solution by vortexing and add 320 µL of this stock solution to the PBS (1×) in the 15 mL PP-tube labelled with “GT1b”.

  **Note**: Alcoholic solution! Careful pipetting recommended.

- Mix the resulting GT1b working solution by vortexing.
- Take a MaxiSorp microplate, and label it with “BP” (for “binding plate”).
- Using a **Combitip**, add 100 µL of PBS (1×) (from the 15 mL PP-tube labelled with “PBS”) to all wells in rows A and B of the microplate (they will serve as control wells for non-specific binding).

- Again using a **Combitip**, add 100 µL of the GT1b working solution (from the 15 mL PP-tube labelled with "GT1b") to all wells in rows C to H of the microplate.

### 2.3.2. Layout for coating the binding plate with GT1b:




- Seal the plate with adhesive foil and incubate in a microplate thermoshaker for 2 hours at 37 °C and 250 rpm.
- (In the meantime, you can start with the preparation of the cleavage plate as described in step 2.6.)

### 2.3.3. Washing the binding plate

- Wash the GT1b-coated binding plate (from step 2.3.1.) 4 times with 300 µL/well of Wash Buffer (from step 2.1.2).

### 2.3.4. Blocking of residual protein binding sites

- Using a multichannel pipette, add 250 µL of Blocking Buffer (from step 2.2.1.) to each well of the binding plate.
- Seal the plate with a fresh sheet of adhesive foil and incubate in a microplate thermoshaker for 2 hours at 37 °C and 250 rpm.
- In the meantime, prepare the toxoid working solution and the TeNT solutions as described in step 2.4.

## 2.4. Preparation of toxoid working solution and TeNT solutions

**Note: Do not vortex solutions containing TeNT or tetanus toxoid!**

### 2.4.1. Preparation of tetanus toxoid working solution (40 Lf/mL)

- Take the tetanus toxoid stock solution out of the refrigerator and warm it to RT.
- Label one 15 mL PP-tube with "toxoid" and add 7 mL Binding Buffer (from step 2.2.2) to this tube.
- Mix the tetanus toxoid stock solution gently with a pipette (e.g. 1000 µL), then add 83 µL of this stock solution to the Binding Buffer in the 15 mL PP-tube labelled with "toxoid" and mix gently by shaking. Return the remaining tetanus toxoid stock solution to the refrigerator (it will be needed again for tests 2-4).

### 2.4.2. Preparation of TeNT solutions

- Thaw one set of TeNT solutions at RT (i.e. one vial each of solutions TeNT 1 to TeNT 6).
- Prepare 3 PP-tubes (50 mL) by filling them up to the 30 mL-mark with absorbent paper as padding material.
- Put 2 of the toxin-containing vials into each 50 mL PP-tube.
- Centrifuge for 1 minute at approximately 84 × *g* in order to collect the solutions at the bottom of the vials. Remove the vials carefully from the 50 mL PP-tubes (to avoid the TeNT solution spreading over the inner surface of the lid again).

- Mix each TeNT solution **gently** with a pipette (e.g. 1000 µL), and store at RT until the blocking incubation of the binding plate (step 2.3.4.) is finished.

## 2.5. Binding of TeNT to the microplate

### 2.5.1. Washing the binding plate

- Wash the GT1b-coated and BSA-blocked binding plate (BP, from step 2.3.4.) 4 times with 300 µL/well of Wash Buffer.

### 2.5.2. Binding of TeNT to the binding plate

- Using a multichannel pipette, add 50 µL Binding Buffer (prepared in step 2.2.2.) to each well of columns 1-6.
- Using a multichannel pipette, add 50 µL tetanus toxoid working solution (40 Lf/mL, prepared in step 2.4.1.) to each well of columns 7-12.

**Layout for the addition of Binding Buffer and toxoid solution to the binding plate:**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | Binding Buffer | | | | | | Tetanus Toxoid working solution | | | | | |
| B | | | | | | | | | | | | |
| C | | | | | | | | | | | | |
| D | | | | | | | | | | | | |
| E | | | | | | | | | | | | |
| F | | | | | | | | | | | | |
| G | | | | | | | | | | | | |
| H | | | | | | | | | | | | |

- Add 50 µL of the TeNT solutions to the appropriate wells of the binding plate:
  - Add 50 µL solution "TeNT 1" to all wells in columns 1 and 12
  - Add 50 µL solution "TeNT 2" to all wells in columns 2 and 11
  - Add 50 µL solution "TeNT 3" to all wells in columns 3 and 10
  - Add 50 µL solution "TeNT 4" to all wells in columns 4 and 9
  - Add 50 µL solution "TeNT 5" to all wells in columns 5 and 8
  - Add 50 µL solution "TeNT 6" to all wells in columns 6 and 7

**Layout for the addition of TeNT solutions to the binding plate:**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | TeNT 1 | TeNT 2 | TeNT 3 | TeNT 4 | TeNT 5 | TeNT 6 | TeNT 6 | TeNT 5 | TeNT 4 | TeNT 3 | TeNT 2 | TeNT 1 |
| B | | | | | | | | | | | | |
| C | | | | | | | | | | | | |
| D | | | | | | | | | | | | |
| E | | | | | | | | | | | | |
| F | | | | | | | | | | | | |
| G | | | | | | | | | | | | |
| H | | | | | | | | | | | | |

- Seal the plate with a fresh sheet of adhesive foil and incubate overnight at 5 ± 3 °C without shaking.

## 2.6. Preparation of the microplate for synaptobrevin cleavage

### 2.6.1. Coating of the cleavage plate with rSyb

- Take one vial of of rSyb stock solution from the freezer (-70 °C) and incubate at RT until the solution has completely thawed, then mix gently with a pipette.
- Add 47 µL rSyb stock solution to 11 mL PBS (1×) (from step 2.1.1.) in a 15 mL PP-tube and mix gently by shaking.
- Take one fresh MaxiSorp microplate, and label it with "CP" (cleavage plate).
- Add 100 µL of the rSyb dilution to **each** well of this plate. (**A Multipette with Combitip should be used for this step.**)
- Seal the plate with a fresh sheet of adhesive foil and incubate for 2 hours at 37 °C, 250 rpm.

### 2.6.2. Blocking of residual protein binding sites

- After the coating incubation described in step 2.6.1., remove the coating solution from the cleavage plate (CP) by aspiration with a washer or, if no washer is available, by inverting the plate and tapping it on absorbent paper to remove all excess liquid.
- **Do not wash** the plate at this step.
- Using a multichannel pipette, add 250 µL of Blocking Buffer (from step 2.2.1.) to each well.
- Seal the cleavage plate with a fresh sheet of adhesive foil and incubate **overnight at 5 ± 3 °C without shaking**.

**Note: Take PIPES Buffer out of the refrigerator and allow it to equilibrate to RT for use on day 2.**

## Day 2: Reduction and cleavage step

## 2.7. Reduction of the bound TeNT

- Preheat a microplate thermoshaker to 37 °C.
- Take the binding plate (BP, from step 2.5.) and the cleavage plate (CP, from step 2.6.) out of the refrigerator and allow them to equilibrate to RT (approx. 30 minutes). In the meantime, prepare the Reduction and Cleavage Buffers as described in steps 2.7.1. and 2.7.2.

### 2.7.1. Preparation of Reduction Buffer

- Take the asolectin stock solution out of the refrigerator.
- Take one vial of the TCEP stock solution out of the freezer and incubate it at RT until the solution has completely thawed, then mix gently with a pipette.
- Combine the following components in a 50 mL PP-tube and mix by vortexing:

  15 mL PIPES Buffer

  + 75 µL asolectin stock solution

  + 75 µL TCEP stock solution

- Transfer the remaining asolectin stock solution to the refrigerator (5 ± 3 °C) (it will be needed again for tests 2-4).

### 2.7.2. Preparation of Cleavage Buffer

- Mix the following components in a 50 mL PP-tube:

  7 mL PIPES Buffer

  + 3.5 g TMAO

- Vortex until the TMAO is completely dissolved (approx. 1 minute).

### 2.7.3. Washing the binding plate

- Take the binding plate (BP) and hold it in a slightly inclined position in order to collect the supernatant in one corner of the wells. Using a multichannel pipette equipped with filter tips, remove the supernatant containing the unbound sample material carefully. Start with row A and then proceed towards row H without changing pipette tips.
- Discard the supernatant into the container for liquid toxin waste.
- Wash the plate 4 times with 300 µL/well of Wash Buffer (from step 2.1.2.).
- Wash the plate once with 300 µL/well of PIPES Buffer using a multichannel pipette. (This extra wash is crucial to remove residual Tween 20, which would inhibit the following protease step).
- Remove PIPES Buffer by aspiration with a washer or, if no washer is available, by inverting the plate and tapping it on absorbent paper to remove all excess liquid.

### 2.7.4. Reduction step

- Add 100 µL Reduction Buffer (from step 2.7.1.) to each well of the binding plate (BP) using a 12-channel pipette. Start with row A and then proceed towards row H.
- Seal the plate with a fresh sheet of adhesive foil and incubate for exactly 30 minutes in a microplate thermoshaker at 37 °C and 250 rpm.
- After approximately 10-15 minutes of this incubation time, start to prepare the cleavage plate as described in step 2.8.1.

## 2.8. Cleavage of rSyb

### 2.8.1. Preparation of the cleavage plate

- Take the rSyb-coated and BSA-blocked cleavage plate (CP).
- Wash the plate 4 times with 300 µL/well of Wash Buffer (from step 2.1.2.).
- Then wash the plate once with 300 µL/well of PIPES Buffer using a multichannel pipette (this extra wash is crucial to remove residual Tween 20, which could inhibit the cleavage reaction).
- Remove PIPES Buffer by aspiration with a washer or, if no washer is available, by inverting the plate and tapping it on absorbent paper to remove all excess liquid.
- Add 50 µL Cleavage Buffer (from step 2.7.2.) to each well of the cleavage plate (CP) using a 12-channel pipette.

### 2.8.2. Cleavage step

- Transfer the supernatants (100 µL) from the binding plate (from step 2.7.4.) to the corresponding wells of the washed cleavage plate (from step 2.8.1.) using a 12-channel pipette adjusted to 100 µL.
- For this transfer, hold the binding plate (BP) in a slightly inclined position (in order to collect the supernatant in one corner of the wells), and carefully position the pipette tips at the edge of the well bottoms in row A.
- Mix the contents of each well by pipetting up and down once with the multichannel pipette, and then transfer the complete supernatant to row A of the cleavage plate (CP).
- Repeat this procedure accordingly for row B and all other rows of the microplate, without changing pipette tips (in order to save time).
- Seal the cleavage plate with a fresh sheet of adhesive foil and incubate for 6 hours in a microplate thermoshaker at 37 °C and 250 rpm.
- The empty binding plate can be discarded.

**Note: Transfer the remaining PIPES Buffer to the refrigerator. It will be needed for tests 2-4.**

## 2.9. Detection of cleaved rSyb

### 2.9.1. Preparation of Antibody Buffer (PBS / 1 % BSA)

- Prepare fresh Antibody Buffer by combining:

  60 mL PBS (1×) (from step 2.1.1.)

  + 0.6 g BSA

- Dissolve using a magnetic stirrer, filtrate using a syringe filter (0.2 µm), and store at RT until use.

### 2.9.2. Incubation with anti-Syb antibody

**Note: Use aseptic technique to keep the supplied antibody sterile!**

- Take the stock solution of the anti-Syb antibody out of the refrigerator.
- Prepare a 1:4000 dilution in a 50 mL PP-tube:

  16 mL Antibody Buffer (from step 2.9.1.)

  + 4 µL anti-Syb antibody

- Return the remaining stock solution of the anti-Syb antibody to the refrigerator immediately (it will be needed again for tests 2-4).
- Store the remaining Antibody Buffer at RT (it will be needed again on day 3).
- Mix the anti-Syb antibody dilution gently using a vortex mixer.
- Wash the cleavage plate (from step 2.8.2.) 4 times with 300 µL/well of Wash Buffer.
- Add 100 µL of the diluted anti-Syb antibody to each well of the microplate.
- Seal the plate with a fresh sheet of adhesive foil and incubate overnight at 5 ± 3 °C without shaking.

**Day 3: Detection**

## 2.10. Incubation with biotin-conjugated Goat anti-Rabbit IgG (secondary antibody)

- Let the cleavage plate (from step 2.9.2.) equilibrate to RT (approx. 30 minutes).
- Take the stock solution of the biotin-conjugated Goat anti-Rabbit IgG (secondary antibody) out of the -25 ± 10°C freezer and warm it to RT, then mix gently with a pipette.
- Prepare a 1:2500 dilution in a 50 mL PP-tube:

  15 mL Antibody Buffer (from step 2.9.1.)

  + 6 µL biotin-conjugated Goat anti-Rabbit IgG

- Return the remaining stock solution of the biotin-conjugated Goat anti-Rabbit IgG antibody to the -25 ± 10 °C freezer immediately (it will be needed again for tests 2-4).
- Mix the biotin-conjugated Goat anti-Rabbit IgG antibody dilution by gently vortexing.
- Wash the cleavage plate 4 times with 300 µL/well of Wash Buffer (from step 2.1.2.).
- Add 100 µL of the biotin-conjugated Goat anti-Rabbit IgG dilution to each well of the plate.
- Seal the plate with a fresh sheet of adhesive foil and incubate for 45 minutes on a microplate shaker at RT with 150-250 rpm.

## 2.11. Incubation with Peroxidase-conjugated streptavidin

- Take the stock solution of the peroxidase-conjugated streptavidin out of the -25 ± 10 °C freezer and warm it to RT, then mix gently with a pipette.
- Prepare a 1:8000 dilution in a 50 mL PP-tube:

16 mL Antibody Buffer

+ 2 µL Peroxidase-conjugated streptavidin

- Return the remaining stock solution of the peroxidase-conjugated streptavidin to the -25 ± 10 °C freezer immediately (it will be needed again for tests 2-4).
- Mix the peroxidase-conjugated streptavidin dilution by gently vortexing.
- Wash the cleavage plate 4 times with 300 µL/well of Wash Buffer (from step 2.1.2).
- Add 100 µL of the diluted peroxidase-conjugated streptavidin to each well.
- Seal the plate with a fresh sheet of adhesive foil and incubate for 45 minutes on a microplate shaker at RT with 150-250 rpm.
- During this incubation, take the TMB solution out of the refrigerator and mix by shaking gently. Prepare one aliquot of 14 mL and equilibrate it to RT **in the dark**. Return the remaining TMB solution to the refrigerator (it will be needed again for tests 2-4).
- Turn on the microplate photometer.

## 2.12. Development with TMB

- Wash the cleavage plate **5 times** with 300 µL/well of Wash Buffer (from step 2.1.2). If using a microplate washer, **set the incubation (soak) time to 10 seconds**.
- Add 100 µL of the TMB solution to each well using a 12-channel pipette. Start with row A and proceed towards row H. Then incubate the plate for **exactly** 25 minutes at RT **in the dark** without shaking.
- Stop the reaction by adding 50 µL/well of 1 M $H_2SO_4$ using a 12-channel pipette. Start with row A and proceed towards row H.

## 2.13. Reading

- Measure the plate immediately in a photometer at 450 nm versus 620 nm as reference wavelength.
- Print out the raw data, and also enter the results of the measurement into the provided Excel result sheet. The instructions and explanations quoted in the reporting sheet should be taken into account.
- Preferably, the results of the plate readings at 450 nm and 620 nm should be reported separately. If your photometer automatically performs the subtraction of the reference measurements and does not allow you to obtain the measurements at both wavelengths separately, it is also possible to report only the differences.

**Note: The remaining Wash Buffer and PBS (1×) should be discarded at the end of each test, and freshly prepared buffers should be used for the next test.**

**Test criteria**

Check after each test if the following criterion (as indicator for a successful test performance) has been fulfilled: When looking at rows C to H of the plate, you should see appreciably higher signals in the wells of column 1 (which contained a high TeNT concentration) than in the wells of column 6 (which contained no TeNT at all).

If this criterion has not been fulfilled, or if you see extremely high background signals (e.g. above 1.0) either in the wells without GT1b (i.e. in rows A and B of the plate) or in any of the wells in column 6, it is recommended that you contact the BSP136 team by e-mail before proceeding with the next test.

## Annex III – Protocol for expression and purification of recombinant synaptobrevin-2 (rSyb)

The rSyb protein for the tetanus BINACLE assay consists of amino acids 1-97 of synaptobrevin-2 from *Rattus norvegicus* (UniProtKB accession number: P63045) fused to an *N*-terminal histidine-tag. Synaptobrevin-2 is also called vesicle-associated membrane protein 2 (VAMP2).

A brief description of the rSyb production process was published in: B. Kegel *et al. Toxicol In Vitro* 2007;21:1641-9.

### Amino acid sequence of the rSyb protein

MGSSHHHHHHSSGLVPRGSHMSATAATVPPAAPAGEGGPPAPPPNLTSNRRLQQTQAQVDEVVDIMRVNVDKVLERDQ KLSELDDRADALQAGASQ*FETSAAKLKRKYWWKNLKMMI

(underlined = amino acids 1-97 of rat synaptobrevin-2; not underlined = His-tag; * = cleavage site used by the TeNT light chain)

### Expression system

A pET15b plasmid (Novagen, Schwalbach, Germany) with an insert encoding the rSyb protein was kindly provided by Dr Thomas Binz (Institute of Biochemistry, Medical School Hannover, Germany).

For rSyb expression, competent *E. coli* BL21 CodonPlus (DE3)-RP cells (Stratagene, LaJolla, USA) are transformed with the pET 15b plasmid encoding rSyb according to the manufacturer's instructions.

### Expression of rSyb

#### Preculture:

- Incubate the *E. coli* BL21 Codon Plus (DE3)-RP bacteria with the pET 15b plasmid encoding rSyb overnight in LB Medium according to Miller (pH 7.5) which has been supplemented with 200 µg/mL carbenicillin and 35 µg/mL chloramphenicol in a shaking incubator at 37 °C and 225 rpm.

#### Protein expression:

- Using a fermenter, add 160 mL of the bacterial preculture to 7.84 litres LB Medium (pH 7.5) supplemented with 200 µg/mL ampicillin and 35 µg/mL chloramphenicol.
- Incubate using the following instrument settings:
    - Stirrer speed: 400 rpm
    - Temperature: 37 °C
    - pH controller: pH 7.4
    - Aeration rate: 4 L/min
- As soon as an optical density (OD) at 600 nm of ~1.0 is reached, add 8 mL of 1 M isopropylthiogalactoside (IPTG) to the culture to induce rSyb expression.
- Five hours later, harvest the cells by centrifugation and store the pellets at -20 °C until further processing.

### Lysis of bacterial cells and preparation of inclusion bodies

- Prepare fresh Lysis Buffer (50 mM Tris, pH 8.0, 25 % *(w/v)* sucrose, 5 mM EDTA, 0.5 mg/mL lysozyme, 250 µg/mL RNaseA, 10 U/mL DNase I, 0.3 mM PMSF, 10 mM benzamidine, 20 µg/mL pepstatin A, 2.5 mM $MgCl_2$).
- Add Lysis Buffer to the frozen bacterial pellets (use 5 mL Lysis Buffer per gram of pellet wet weight) and incubate for 1 hour at 37 °C under constant agitation (200 rpm). Then resuspend the pellets using a 10 mL pipette, and incubate again for 1 hour at 37 °C and 200 rpm.
- Treat the suspension with an ultrasonic homogeniser (2 cycles of 15 minutes each, instrument settings: 50 watts, 50 %). Keep the tube on ice during sonication to prevent inappropriate heating.

- After sonication, centrifuge the suspension for 50 minutes at 4 °C and 32 000 × *g*. The resulting pellets contain the inclusion bodies.
- Wash these pellets three times in PBS (phosphate-buffered saline without $Ca^{2+}$ and $Mg^{2+}$ pH 7.4) with decreasing Triton X-100 concentrations:
    - 1st washing step: use PBS with 1 % Triton X-100
    - 2nd washing step: use PBS with 0.5 % Triton X-100
    - 3rd washing step: use PBS without Triton X-100
- After each washing step, centrifuge the suspension for 50 minutes at 32 000 × *g* and 4 °C.
- After the final washing step, the pellets containing the inclusion bodies can be stored at -20 °C until the FPLC purification is started.

## Purification of rSyb2 using FPLC

- Add freshly prepared Urea Buffer (8 M urea, 0.1 M $NaH_2PO_4$, 0.01 M Tris, pH 8.0) to the pellets containing the inclusion bodies. Use the same buffer volume as in the lysis step.
- Resuspend the inclusion body pellets in the Urea Buffer (pH 8.0) using a 10 mL pipette, then agitate the solution for 2 h at room temperature and 250 rpm.
- Centrifuge for 60 minutes at 26 500 × *g* and 4 °C to remove non-dissolved material.
- Apply the supernatant containing the solubilised inclusion bodies to an FPLC column:
    - Column material: Ni-NTA Superflow (Qiagen, Hilden, Germany)
    - Column size: 25 mL
    - Flow rate: 1 mL/minute
    - Other column materials intended for purifying His-tagged proteins as well as other column sizes and flow rates may also be suitable.
- Wash the column using Urea Buffers (8 M urea, 0.1 M $NaH_2PO_4$, 0.01 M Tris) with gradually decreasing pH values:
    - First, add 3 column volumes (e.g. 75 mL) of Urea Buffer, pH 8.0
    - Then, add 3 column volumes (e.g. 75 mL) of Urea Buffer, pH 6.5
    - Afterwards, add 4 column volumes (e.g. 100 mL) of Urea Buffer, pH 5.9
- Elute the rSyb protein by adding 5 column volumes of Urea Buffer (8 M urea, 0.1 M $NaH_2PO_4$, 0.01 M Tris) that has been adjusted to pH 4.5.
- Collect the eluted material in fractions. The fraction size should approximately correspond to 0.5 column volumes, e.g. 15 mL fractions can be collected if a 25 mL column is used.
- Check the eluted fractions for their rSyb content by SDS-PAGE on 15 % polyacrylamide gels, and pool the fractions containing high amounts of pure rSyb.

## Dialysis, protein determination and storage

- Using a dialysis tubing with a molecular weight cut-off of 3500 Da, dialyse the pooled eluate fractions against 20 mM sodium acetate, pH 4.5 with 0.05 % Triton X-100 in three rounds (first round: 2 hours, second round: overnight, third round: 2 hours).
- After dialysis, determine the protein concentration of the rSyb solution by means of the DC Protein Assay (Bio-Rad Laboratories GmbH, Feldkirchen, Germany).

Finally, aliquot the final rSyb solution, shock-freeze the aliquots in liquid nitrogen and store them at -80 °C.

## Annex IV – Supplemental study results

Table 1 – *Median absorbance values for each of the tested TeNT concentrations in binding buffer (BB) and toxoid (Txd)*

| Laboratory | N | Condition | 0 ng/mL | 0.11 ng/mL | 0.33 ng/mL | 1 ng/mL | 3 ng/mL | 9 ng/mL |
|---|---|---|---|---|---|---|---|---|
| 1 | 24 | BB | 0.38 | 0.40 | 0.48 | 0.68 | 1.18 | 2.38 |
| | 24 | Txd | 0.38 | 0.41 | 0.47 | 0.69 | 1.29 | 2.38 |
| 2 | 24 | BB | 0.12 | 0.18 | 0.26 | 0.55 | 1.29 | 2.57 |
| | 24 | Txd | 0.11 | 0.19 | 0.25 | 0.54 | 1.17 | 2.82 |
| 3 | 18* | BB | 0.12 | 0.18 | 0.31 | 0.69 | 1.57 | 2.97 |
| | 18* | Txd | 0.12 | 0.17 | 0.30 | 0.67 | 1.64 | 3.01 |
| 4 | 24 | BB | 0.12 | 0.15 | 0.21 | 0.40 | 0.94# | 1.82 |
| | 24 | Txd | 0.11 | 0.15 | 0.22 | 0.43 | 0.93 | 1.81 |
| 5 | 24 | BB | 0.29 | 0.42 | 0.67 | 1.19 | 2.52 | 3.73‡ |
| | 24 | Txd | 0.28 | 0.40 | 0.61 | 1.31 | 2.58 | 3.73 |
| 6 | 24 | BB | 0.22 | 0.30 | 0.47 | 0.95 | 2.04 | 3.52 |
| | 24 | Txd | 0.22 | 0.29 | 0.43 | 0.82 | 1.90 | 3.35 |
| 7 | 36$ | BB | 0.28 | 0.43 | 0.68 | 1.29 | 2.35 | 2.88 |
| | 36$ | Txd | 0.29 | 0.43 | 0.69# | 1.32 | 2.31 | 2.88 |
| 8 | 18* | BB | 0.19 | 0.23 | 0.33 | 0.65# | 1.34 | 2.87 |
| | 18* | Txd | 0.20 | 0.24 | 0.35 | 0.62 | 1.47 | 2.68 |
| 9 | 24 | BB | 0.21 | 0.28 | 0.41 | 0.69# | 1.49 | 2.80† |
| | 24 | Txd | 0.19 | 0.26 | 0.37 | 0.69 | 1.78 | 2.99 |

* One assay was excluded,
$ two additional assays were performed,
# one atypical value was removed,
† two atypical values were removed,
‡ nine observations were not taken into account due to photometer saturation.

Table 2 – *Calculation of the intra-laboratory variation based on the RP estimates*

| Laboratory | Assay | $ED_{50}$ values [ng/$ED_{50}$] | | RP | GM | GCV |
|---|---|---|---|---|---|---|
| | | Binding Buffer | Toxoid | | | |
| 1 | 1 | 4.21 | 3.94 | 0.94 | 0.99 | 9 % |
| | 2 | 3.29 | 3.56 | 1.08 | | |
| | 3 | 4.01 | 3.61 | 0.90 | | |
| | 4 | 5.67 | 5.92 | 1.04 | | |
| 2 | 1 | 4.46 | 5.05 | 1.13 | 1.02 | 16 % |
| | 2 | 2.38 | 2.04 | 0.86 | | |
| | 3 | 1.44 | 1.33 | 0.92 | | |
| | 4 | 7.97 | 9.60 | 1.21 | | |
| 3 | 1 | 3.06 | 2.87 | 0.94 | 1.02 | 12 % |
| | *2 (excl)* | *inv.* | *inv.* | *inv.* | | |
| | 3 | 0.51 | 0.60 | 1.17 | | |
| | 4 | 1.04 | 1.02 | 0.98 | | |
| 4 | 1 | 4.55 | 3.25 | 0.72 | 0.93 | 20 % |
| | 2 | 2.89 | 2.68 | 0.93 | | |
| | 3 | 2.06 | 2.38 | 1.16 | | |
| | 4 | 1.71 | 1.64 | 0.96 | | |
| 5 | 1 | 1.02 | 1.04 | 1.02 | 1.02 | 3 % |
| | 2 | 0.76 | 0.77 | 1.01 | | |
| | 3 | 0.49 | 0.49 | 1.00 | | |
| | 4 | 0.65 | 0.69 | 1.06 | | |
| 6 | 1 | 1.23 | 1.50 | 1.22 | 1.22 | 10 % |
| | 2 | 1.42 | 1.55 | 1.09 | | |
| | 3 | 0.92 | 1.10 | 1.20 | | |
| | 4 | 3.91 | 5.39 | 1.38 | | |
| 7 | 1 | 0.43 | 0.44 | 1.02 | 0.99 | 5 % |
| | 2 | 0.55 | 0.50 | 0.91 | | |
| | 3 | 0.60 | 0.59 | 0.98 | | |
| | 4 | 0.58 | 0.61 | 1.05 | | |
| | 5 | 0.52 | 0.51 | 0.98 | | |
| | 6 | 0.47 | 0.46 | 0.98 | | |
| 8 | *1(excl.)* | *14.4* | *15.3* | *1.06* | *1.02* | 5 % |
| | 2 | 1.90 | 1.87 | 0.98 | | |
| | 3 | 2.81 | 2.83 | 1.01 | | |
| | 4 | 1.52 | 1.64 | 1.08 | | |
| 9 | 1 | 1.77 | 2.26 | 1.28 | 1.04 | 19 % |
| | 2 | 1.59 | 1.54 | 0.97 | | |
| | 3 | 6.14 | 5.03 | 0.82 | | |
| | 4 | 1.57 | 1.79 | 1.14 | | |

Laboratory 3, Assay 2 and Laboratory 8, Assay 1 have been excluded from calculations and are shown for information only (*red italic font*). inv. = invalid

Table 3 – *Mean and SD of the blank controls and the resulting cut-off absorbance values per assay*

| | $Mean_{BLK}$ | | | | $SD_{BLK}$ | | | | Cut-off Values | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Assay | | | | Assay | | | | Assay | | | |
| Lab | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 |
| 1 | 0.414 | 0.394 | 0.284 | 0.357 | 0.020 | 0.014 | 0.016 | 0.022 | 0.481 | 0.440 | 0.338 | 0.430 |
| 2 | 0.103 | 0.207 | 0.131 | 0.089 | 0.004 | 0.009 | 0.005 | 0.004 | 0.114 | 0.236 | 0.146 | 0.101 |
| 3 | 0.118 | *0.166* | 0.130 | 0.109 | 0.012 | *0.018* | 0.012 | 0.007 | 0.157 | *0.226* | 0.171 | 0.132 |
| 4 | 0.111 | 0.129 | 0.151 | 0.103 | 0.005 | 0.011 | 0.008 | 0.008 | 0.127 | 0.167 | 0.178 | 0.129 |
| 5 | 0.297 | 0.281 | 0.158 | 0.315 | 0.010 | 0.016 | 0.005 | 0.019 | 0.329 | 0.334 | 0.175 | 0.377 |
| 6 | 0.240 | 0.217 | 0.204 | 0.220 | 0.011 | 0.007 | 0.022 | 0.011 | 0.277 | 0.241 | 0.278 | 0.257 |
| 7 | 0.309 | 0.287 | 0.275 | 0.264 | 0.009 | 0.011 | 0.006 | 0.005 | 0.338 | 0.325 | 0.294 | 0.279 |
| | | | 0.283* | 0.296# | | | 0.008* | 0.007# | | | 0.310* | 0.318# |
| 8 | *0.483* | 0.185 | 0.170 | 0.303 | *0.047* | 0.019 | 0.021 | 0.020 | *0.638* | 0.248 | 0.239 | 0.368 |
| 9 | 0.202 | 0.207 | 0.164 | 0.213 | 0.017 | 0.032 | 0.020 | 0.022 | 0.258 | 0.312 | 0.230 | 0.284 |

Values in *red italic* font are excluded from the calculations and shown only for information (i.e. Assays 2 and 1 by Laboratories 3 and 8, respectively).
*,# Laboratory 7, Assays 5 and 6, respectively.

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