Collaborative study for the validation of cell line assays for in-process toxicity and antigenicity testing of *Clostridium septicum* vaccine antigens – Part 2: Optimisation of cell line assays

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ABSTRACT

During the production of clostridial vaccines large numbers of mice are used for various in-process control tests. Replacement in vitro assays had been developed for the testing of the toxins and toxoids of several clostridial species, but none of these assays had been assessed in an international collaborative study. Under the common aegis of the European Partnership for Alternative Approaches to Animal Testing (EPAA) and of the European Directorate for the Quality of Medicines & HealthCare (EDQM), a project on clostridial vaccines for veterinary use was started as part of the EDQM-co-ordinated Biological Standardisation Programme (BSP). Within the framework of this project (coded BSP130) a collaborative study was organised to evaluate Vero cell-based alternative methods to the current mouse tests used to measure: i) the toxicity of Clostridium septicum toxin, ii) the absence of toxicity of C. septicum toxoid and iii) the antigenicity of C. septicum toxoid. The principal aims of the study were to determine the repeatability and reproducibility of the in vitro assays and to demonstrate concordance of the in vitro and current in vivo tests.

The study results demonstrated good concordance, but the information gathered through the study (later on called Part 1) and the participants' workshop prompted the extension of the project in order to further optimise the in vitro protocols and improve their repeatability and re-producibility, which were comparable to but not better than those of the in vivo assays in Part 1. The 3 in vitro assays to be optimised in the extension of the BSP130 project were : i) the in vitro toxin neutralisation equivalence plus (TNE+), as a replacement for the in vivo minimum lethal dose (MLD) test for quantification of the toxicity of toxin; ii) the in vitro MLD, as a replacement for the in vivo MLD test for detection of residual toxicity associated with toxoid; iii) the in vitro total combining power (TCP), as a replacement for the in vivo TCP test for quantification of the antigenicity of toxoid. At this point, the Analytical Method Transfer Laboratory of Ceva-Phylaxia (Hungary), supported by the project management team, developed suitable SOPs for the 3 in vitro assays. These optimised methods were further assessed in BSP130 through a second international collaborative study (Part 2) aimed at defining repeatability and reproducibility in

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different laboratories and determining the levels of improvement compared with the original in vivo tests and the initial in vitro assays used in Part 1 of the project.

Fourteen laboratories, comprising 4 public sector and 10 manufacturers' medicines control laboratories, from 11 countries participated in the collaborative Part 2 study, each testing 6 different C. septicum toxins and 6 C. septicum toxoids. Improved repeatability and reproducibility were observed for the optimised assays. The results of this study confirm the suitability of these assays for in-process control of C. septicum vaccines, with better repeatability and reproducibility than their in vivo equivalents. It is expected that, with appropriate minor changes and the use of relevant reagents, these optimised in vitro assays could be used not only for the assessment of C. septicum toxins and toxoids but for all cytotoxin-based clostridial antigens. The development and implementation of such in vitro assays would offer a great opportunity to significantly reduce animal usage, shorten the duration of QC test procedures and increase the precision of toxicity and antigenicity assays in clostridial veterinary vaccine in-process control. This would also provide more accurate and reproducible dosing of antigens in the final vaccine products, help to promote compendial acceptance and to proffer a basis for improved international harmonisation across this area of product testing.

KEYWORDS

Clostridium septicum vaccine, minimum lethal dose, residual toxicity, total combining power, European Partnership for Alternative Approaches to Animal Testing, Biological Standardisation Programme, EDQM, cell-based assay, toxicity testing, antigenicity testing, *in vivo* test, *in vitro* test, 3Rs, Council of Europe, European Pharmacopoeia.

1. INTRODUCTION

Vaccines for protection against diseases caused by clostridial species in animals are widely used. Their pharmaceutical quality is controlled by vaccine manufacturers in accordance with the specifications of the European Pharmacopoeia (Ph. Eur.) monographs for clostridial veterinary vaccines and with their market authorisation dossiers. It has long been known that large numbers of mice are used in testing during the production of clostridial vaccines [1] and this became a concern to the veterinary vaccines industry which addressed the issue by creating the INVITRO consortium at the end of the 1990s [2]. In addition, private and public sector control laboratories [3-9] committed to the 3Rs (Replacement, Reduction and Refinement [10]) in clostridial veterinary vaccine control and attempted to develop potential alternatives. This led to the start of several European projects run by the European Directorate for the Quality of Medicines & HealthCare (EDQM, Council of Europe) within the framework of the Biological Standardisation Programme (BSP). These projects, focusing on the establishment of a serological potency assay to replace *in vivo* toxin neutralising testing (TNT) [11-14], resulted in the establishment of 3 Ph. Eur. Biological Reference Preparations (BRPs): the Clostridia (multi-component) rabbit antiserum (for vaccines – vet. use) BRP, the *Clostridium tetani* guinea pig antiserum (for vaccines – vet. use) BRP and the *Clostridium tetani* rabbit antiserum (for vaccines – vet. use) BRP.

However, for many clostridial vaccines both the toxin and toxoid bulk (obtained by detoxification of the toxin and used to formulate the final vaccine batches) are still currently controlled by animal-based tests. The in-process controls for toxicity and antigenicity are performed in mice using the minimum lethal dose (MLD) and the total combining power (TCP) tests, respectively. As these tests account for the use of large numbers of animals [1], new, *in vitro* methods to replace them are highly desirable. In addition, because of their potentially higher sensitivity and precision, *in vitro* assays may offer better tracking of production consistency and allow more accurate vaccine blending.

Preliminary research studies indicated that cell line assays could replace mouse tests for certain in-process testing of bulk clostridial toxins and/or toxoids and replacement assays were developed for the testing of vaccines derived from several clostridial species [15] but none of

these assays had been assessed in an international collaborative study. Therefore a project was initiated by the European Partnership for Alternative Approaches to Animal Testing (EPAA) as part of the project "Application of the 3Rs and Consistency Approach for Improved Vaccine Quality Control/Novel *in vitro* methods to replace animal-based in-process control tests" [16] to evaluate Vero cell (VC)-based alternative methods to the current mouse tests used to measure the toxicity of *Clostridium septicum* toxin (the MLD test), the residual toxicity of *C. septicum* toxoid using the MLD test setup and the antigenicity of *C. septicum* toxoid (the TCP test). The resulting project was also taken up by the EDQM within the framework of its BSP [17]). The EDQM organised and co-ordinated the joint project (referred to as BSP130) as an international collaborative study in 2 phases. The principal aims of BSP130 Phases 1 and 2 (later referred to as BSP130 Part 1) were to determine the repeatability and reproducibility of the *in vitro* assays and to demonstrate concordance of the *in vitro* and current *in vivo* TCP and MLD tests [18]. To facilitate these comparisons, the methodologies used for the *in vitro* assays were essentially identical to those of the *in vivo* tests, with the exception that the final processed samples were assessed using VCs rather than mice.

The results of BSP130 Part 1 [18] for the repeatability and reproducibility of the *in vitro* VC-based assays were comparable to, but not much better than, those of the *in vivo* assays. However, the *in vitro* assays were easily transferable to other laboratories, and the concordance between the *in vivo* and *in vitro* methods at the level of the grand mean of all laboratories' results was excellent, thus supporting the assumption that the VC assays (VCAs) could be used as alternatives to the mouse tests for the assessment of *C. septicum* toxin MLD and toxoid TCP values. Further analysis suggested that with a protocol optimised for the *in vitro* assays alone, it should be possible to establish improved assays. This analysis was presented at a BSP130 participants' workshop [19] and the conclusions were endorsed, resulting in an extension of the project, referred to as BSP130 Phase 3. Such assays were developed at Ceva-Phylaxia under the direction of the project management team, and a second collaborative study, herein referred to as BSP130 Phase 3 (and later also as BSP130 Part 2) was undertaken, again co-ordinated by the EDQM in collaboration with the EPAA, which provided financial support and scientific advice through their projects "Application of the 3Rs and Consistency Approach for Improved Vaccine Quality Control" [20] and "Clostridial vaccines". The present publication describes the results obtained in Phase 3 with 3 new optimised *in vitro* assays that were devised to replace the *in vivo* MLD tests for assessment of toxicity of toxin, the *in vitro* MLD test and for detection of residual toxicity associated with toxoids, and the *in vivo* TCP test for quantification of toxoid antigenicity.

Dr Keith Redhead, Dr Lukas Bruckner and Dr Botond Siklodi were nominated by the BSP steering committee as project co-leaders. Dr Marlies Halder (EURL ECVAM) and Dr Irene Manou were nominated by the EPAA project platform as project leader and scientific advisor, respectively. Dr Marie-Emmanuelle Behr-Gross acted as the study co-ordinator on behalf of the two organisations. The collaborative study aims were to assess the performance of optimised *in vitro* methods, based on the previously validated VCA, for use as alternative methods to the *in vivo* (mouse) tests used during the production of toxoid based *C. septicum* vaccine antigens. As VCAs had already been demonstrated to be suitable replacements for the *in vivo* tests in Part 1 of the study [18], BSP130 Part 2 required only *in vitro* testing.

The precision of the optimised VCA was studied by obtaining information on intra-laboratory variation (repeatability) and on inter-laboratory variation (reproducibility). To confirm the appropriateness of the test methods and settings, and to allow the characterisation of the test toxins and toxoids, the study was divided into four consecutive steps:

Step I: confirmation of sensitivity of the participants' VC lines was performed by each laboratory to verify that their cell line was appropriately susceptible to the toxicity of *C. septicum* toxin.

Step II: confirmation that the participants' VC lines showed suitably limited sensitivity to any latent toxic effects of the standard antitoxin and test toxoids.

Step III: measurement of the toxin neutralisation equivalence plus (TNE+) of the detector toxin was performed in participating laboratories.

Step IV: testing of the BSP130 Phase 3 sample panels (toxins and toxoids) was performed in the participating laboratories using the appropriate assay protocols (i.e. *in vitro* TNE+, MLD or TCP assays).

Depending on the results of the study, this approach – with appropriate modifications – could be extended to all cytotoxin-based clostridial antigens used in veterinary vaccination.

2. AIMS OF THE STUDY

Currently *in vivo* MLD and TCP tests are used to assess the quality of the active component(s) at various points during the production of *C. septicum* toxoid-based antigens. At the termination of fermentation the MLD test (LD_{50} or L+ test for some manufacturers) is used to measure the toxicity of the toxin on the basis of how much it can be diluted while retaining lethality for mice. During and at the end of toxoiding, the MLD test is used to monitor the level of residual toxicity associated with the toxoid, again on the basis of lethality in mice. On completion of toxoiding, the TCP test is used to quantify the antigenicity of the toxoid. This is based on the amount of a reference preparation of neutralising antitoxin that is bound by the toxoid. The amount of antitoxin bound is measured by checking whether the residual unbound antitoxin is sufficient to neutralise a set amount of a specified *C. septicum* detector toxin. Unbound detector toxin is then assessed by lethality in mice. Three optimised assays were developed for Phase 3 of BSP130. These were:

1. In vitro TNE+ as a replacement for the *in vivo* MLD test for quantification of the toxicity of toxin. In Part 1 [18], *in vitro* MLD was used for the quantification of the toxicity of toxin for the sake of comparability with the *in vivo* method. However, the *in vitro* MLD results were strongly dependent on the highly variable sensitivity of the cell lines used by different labs. In order to alleviate this dependency, the TNE+ assay was introduced in Phase 3 and was the *in vitro* equivalent of the $L+_{50}$ test in mice. In the TNE+ assay, the toxicity of toxin was quantified on the basis of the dilution rate at which its cytotoxicity is in equilibrium with the neutralisation capacity of a standard antitoxin calibrated in International Units (IU). Excess toxicity was assessed on the basis of lethality in VCs. The last dilution still showing cytotoxicity after pre-incubation with a set amount of standard antitoxin is the basis of TNE+ determination. This assay can generate absolute rather than relative values for the toxicity of a toxin.
2. In vitro MLD, as a replacement for the *in vivo* MLD test for detection of residual toxicity associated with toxoids. In this assay, MLD was used because cell line sensitivity is not critical/important for this purpose. Residual toxicity was measured on the basis of how much the studied toxoid had to be diluted before loss of lethality in VCs. The results were expressed as a titre.
3. *In vitro* TCP, as a replacement for the *in vivo* TCP test for quantification of the antigenicity of toxoid. In this assay, the antigenicity of toxoid was measured on the basis of the amount of a standard neutralising antitoxin calibrated in IU that was bound by the toxoid. The amount of antitoxin bound was measured by checking whether residual unbound antitoxin was sufficient to neutralise a specified amount of detector toxin. Unbound detector toxin was then assessed by lethality in VCs. The results were expressed in TCP units.

The ultimate goals of the study were to demonstrate the suitability of the three optimised proposed replacement methods by evaluating their intra- and inter-laboratory variation (repeatability and reproducibility, respectively) in the participating laboratories.

3. PARTICIPANTS

In total, 14 laboratories from 10 countries participated in the collaborative study, including 4 public laboratories (Official Medicines Control Laboratories; OMCLs) and other public institutions and 10 manufacturers. A list of participants is given in section 9. Each laboratory is referred to in this report by an arbitrarily assigned number not necessarily representing the order of the listing.

4. MATERIALS, METHODS AND STUDY DESIGN

4.1. Materials provided centrally

Each participating laboratory was provided with 6 test toxins, 6 test toxoids, the standard antitoxin and the detector toxin.

4.1.1. *C. septicum* toxins

C. septicum toxin batches donated by veterinary vaccine manufacturers and originating from different production sites were used in the study. Six samples of differing toxicities coded TxR, TxS, TxV, TxW, TxY and TxZ were distributed to the study participants. TxY and TxZ were obtained by dilution of the detector toxin CSTx2 (1.2-fold and 3-fold dilutions, respectively). Details of the recommended dilution ranges for these toxin samples are indicated in Annex 2. The samples were supplied as frozen solutions, shipped on dry ice.

4.1.2. *C. septicum* toxoids

Six batches of *C. septicum* toxoid (coded TdA, TdC, TdD, TdN, TdO and TdP), obtained from various manufacturers and production sites and of differing antigenicities, were used in the study. Details of the recommended dilution ranges for these toxoids are indicated in Annex 2. The samples were supplied as solutions, shipped at +2 to 8 °C.

4.1.3. Standards and critical reagents

Standard antitoxin: *Clostridium septicum* (gas gangrene) antitoxin (coded VI, Ref), equine, 3rd International Standard (IS) (https://www.nibsc.org/documents/ifu/VI.pdf) with defined activity of 500 IU/ampoule. The antitoxin was supplied as a freeze-dried powder and shipped at +2 to 8 °C. Detector toxin: *Clostridium septicum* detector toxin (coded CSTx2). The detector toxin was produced and tested in the *in vivo* (mouse) L+ and LD_{50} assays by the manufacturer. Its quality fulfilled the requirements described in Ph. Eur. monograph 0364 *Clostridium septicum* vaccine for veterinary use: "… not less than 10 LD_{50} in each L+/5 dose". The toxin was supplied as a frozen solution and shipped on dry ice.

4.1.4. Storage conditions and use of test samples and reference reagents provided centrally

TOXINS

All test and reference toxins were delivered as sterile frozen aliquots of approximately 12 mL and 25 mL each, respectively. Upon receipt, the aliquots were to be stored below −15 °C. Prior to the start of testing, participants were to prepare 0.5 mL aliquots and store them frozen at less than −15 °C.

When ready for testing, one 0.5 mL aliquot of toxin was to be allowed to thaw at 2 to 8 °C prior to use. All manipulations of the toxins were to be performed under sterile conditions and the toxin aliquots were to spend the minimum amount of time at temperatures above 8 °C. Once a toxin aliquot had been thawed, any toxin remaining at the end of the day was to be discarded.

TOXOIDS

All test toxoids were delivered as sterile chilled (2 to 8 °C) aliquots of approximately 12 mL each. Upon receipt, the aliquots were to be stored at 2 to 8 °C. Prior to the start of testing, participants were to prepare 0.5 mL aliquots and store them at 2 to 8 °C.

When ready for testing, one 0.5 mL aliquot of toxoid was used at a time. All manipulations of the toxoids were to be performed under sterile conditions and the toxoid aliquots were to spend the minimum amount of time at temperatures above 8 °C. Once an aliquot of toxoid had been sampled, any toxoid remaining at the end of the day was to be discarded.

C. SEPTICUM STANDARD ANTITOXIN (VI)

The following procedures were to be performed under sterile conditions. Once the ampoule of *C. septicum* standard antitoxin (VI) had been opened it was to be initially rehydrated with 1.0 mL of sterile distilled water or equivalent and mixed thoroughly as indicated in the leaflet provided by the custodian laboratory. This material was then further diluted with 9.0 mL of sterile physiological saline to give 10.0 mL of solution containing 50 IU/mL. This solution was then aliquoted into 10 volumes of 1.0 mL and stored at less than −15 °C until needed.

For TCP assays and the TNE+ determination, where performed, 1.0 mL aliquots of the antitoxin were thawed and diluted to 5 IU/mL by the addition of 9.0 mL of sterile nutrient broth saline (NBS). A 3.0 mL portion of the 5 IU/mL solution was retained for use in the toxicity determination of the detector toxin. To the remaining 7.0 mL of the solution was added 1.75 mL of sterile NBS to give 8.75 mL of 4 IU/mL for use in the *in vitro* TCP determinations. Any variations from this approach were to be detailed and reported.

4.2. Methods and study design

4.2.1. Methods

The methods to be used in this collaborative study were *in vitro* MLD, *in vitro* TNE+ and *in vitro* TCP. These assays were to be performed using the Standard Operating Procedures (SOPs) provided by the study organisers (Annexes 3–5). For each method, data interpretation, validity criteria and calculation methods for test results are also described in the corresponding Annex.

In vitro VC MLD assay. The methodology provided in the study protocol (Annex 3) was used to perform the assay using the detector toxin (CSTx2) in order to confirm the sensitivity of each participant's VC line and on the test toxoids and standard antitoxin to establish levels of latent toxicity. In each case, the aim was to complete 1 valid assay. However, the results from all of the assays performed had to be submitted.

In vitro VC TNE+ assay. The methodology provided in the study protocol (Annex 4) was used to perform the assay using the detector toxin in order to determine its TNE+ value. The results of 1 valid assay performed using the 1.3-fold dilution series as a "range finding" assay and 3 valid assays performed using the 1.1-fold dilution series were to be reported. However, the results from all of the assays performed had to be submitted.

The assay was also used to measure the toxicity of the test toxins. The results of 1 valid assay performed using the 2-fold dilution series as a "range finding" assay and 3 valid assays performed using the 1.3-fold dilution series for each test toxin were to be reported. However, the results from all of the assays performed had to be submitted.

In vitro VC TCP assay. The methodology provided in the study protocol (Annex 5) was used to measure the antigenicity of the test toxoids. The results of 1 valid assay performed using the 20-increment step dilution series as a "range finding" assay and 3 valid assays performed using the 10-step dilution series for each test toxoid were to be reported. However, the results from all of the assays performed had to be submitted.

4.2.2. Study design

TRAINING STEP DESIGN

Although some of the participants had been involved in Part 1, the TNE+ and *in vitro* TCP assays to be performed in Part 2 were completely novel for almost all of them. Therefore, it was considered that it would be advantageous for the participants to perform at least 1 TNE+ assay and 1 TCP assay, for training purposes, prior to beginning the actual study testing. The detector toxin (CSTx2) and test toxoid TdA were to be used in 1.3-fold serial and 20-increment step dilutions, respectively, in this training section according to the protocols provided by the study organisers (Annexes 4 and 5).

COLLABORATIVE STUDY DESIGN

The experimental phase of the collaborative study was divided into 4 steps, to be run successively as shown in Table 1.

Table 1 – *Overall experimental design of BSP130 Part 2*

Step	I	II		III		IV			
Assay	MLD	MLD	MLD	TNE+	TNE+	TNE+	TNE+	TCP	TCP
Test materials	CSTx2	All test toxoids	Antitoxin (VI)	CSTx2	CSTx2	All test toxins	All test toxins	All test toxoids	All test toxoids
Purpose	Determination of VC sensitivity	Determination of latent toxicity	Determination of latent toxicity	TNE+ range finding assay	Determination of TNE+ value	TNE+ range finding assay	Determination of TNE+ values	TCP range finding assay	Determination of TCP values
SOP	Annex 3			Annex 4			Annex 5		
Details	2-fold dilution series from 1 in 8000 to 1 in 4 096 000	2-fold dilution series from 1 in 5 to 1 in 2560	2-fold dilution series from 5 IU/mL to 0.00975 IU/mL	10 step 1.3-fold dilution series, as detailed in the protocol	10 step 1.1-fold dilution series, as appropriate	10 step 2-fold dilution series, as detailed in the protocol	10 step 1.3-fold dilution series, as appropriate	20-increment step dilution range, as detailed in the protocol	10-increment step dilution range, as appropriate
No. of valid assays	1	1	1	1	3	1	3	1	3

4.2.2.1. STEP I: ASSESSMENT OF SENSITIVITY OF CELL LINES IN THE *IN VITRO* MLD

The sensitivity of each participant's VC line to *C. septicum* toxin was assessed in the *in vitro* MLD test using CSTx2. This toxin was handled as described in section 4.1.4 and then subjected to a pre-dilution of 8000-fold in NBS. This was followed by nine 2-fold dilutions, using NBS, to give a range from 1 in 8 000 to 1 in 4 096 000. This dilution series was assessed in 6 replicate rows of VCs (see plate design in Annex 3, Table 3) which were then monitored for lethal effects of the toxin.

Provided the end-point fell within the expected range the laboratory could start Step II of the study.

4.2.2.2. STEP II: LATENT TOXICITY ASSESSMENT OF STANDARD ANTITOXIN AND TEST MATERIALS IN THE *IN VITRO* MLD TEST

The standard *C. septicum* antitoxin (VI) was to be reconstituted, diluted and stored as described in section 4.1.4. It was then further diluted with NBS to a concentration of 5 IU/mL. Each of the 6 *C. septicum* test toxoids was diluted 1 in 5 in NBS. All 6 toxoids and the standard antitoxin were then to undergo nine 2-fold dilutions, using NBS, to give a total of 10 dilutions of each material. Each dilution series was assessed in a row of VCs (see plate design in Annex 3, Table 4) which were then monitored for lethal effects due to non-specific toxicity or to residual toxin, respectively.

Once the latent toxicity testing was finished and the results reported, each laboratory could continue with Step III of the study.

4.2.2.3. STEP III: ASSESSMENT OF TNE+ VALUE FOR DETECTOR TOXIN (CSTX2)

The toxicity of the detector toxin (CSTx2) was assessed in the *in vitro* TNE+ assay. This toxin was handled as described in section 4.1.4 and then diluted to generate a 10 step 1.3-fold dilution series of CSTx2. The dilution range provided in Annex 2 was used for the 1.3-fold dilutions. Each of the 10 dilutions was assessed in 2 sets of 6 parallel rows of VCs on 2 replicate plates (see plate design in Annex 4, Table 6), which were then monitored for lethal effects.

The next step was to refine the TNE+ value of CSTx2 by repeating the assay at 1.1-fold steps, starting at the penultimate dilution giving the last positive response in all 6 wells in the 1.3-fold

step assay. For the determination of the penultimate dilution for the 1.1-fold assay, the lowest end-point value in the six 1.3-fold step parallels was to be used if the results were not the same in all 6 rows. This assay was to be repeated until 3 valid assays had been obtained and the results reported. The geometric mean (GM) of the 3 assay results was to be used for CSTx2 in the participant's specific system for TCP testing in Step IV. Once the TNE+ value for CSTx2 testing was obtained and the results reported, each laboratory could continue with Step IV of the study.

4.2.2.4. STEP IV: ASSESSMENT OF TEST MATERIALS BY *IN VITRO* TNE+ AND TCP

Each of the 6 test toxins and 6 toxoids provided was tested in the appropriate *in vitro* assay (TNE+ assay for toxins or TCP assay for toxoids). The test samples were handled as described in section 4.1.4 and then diluted as prescribed thereafter and using information provided in Annex 2 (dilution ranges obtained from one laboratory on one occasion). They were not necessarily the most appropriate dilution ranges for all of these materials in every participant's testing system. Therefore participants were allowed to adapt the dilution ranges used to their settings.

Each independent TNE+ or TCP assay was performed on duplicate plates tested in parallel and was repeated on different days, until a minimum of 3 valid assays had been obtained for each test material. In the case of assays that were partially invalid, only the materials for which invalid results were obtained needed to be subjected to additional assays. All the results, including those from invalid tests, were recorded and reported.

In vitro TNE+ assay

The TNE+ assay was performed according to the methodology provided in Annex 4.

For each test toxin, the indicative dilution range provided in Annex 2 was used for the 2-fold dilutions. To each of these 10 dilutions was added an equal volume of the standard antitoxin (VI) diluted to a concentration of 0.2 IU/mL. Each of the 10 mixtures was assessed in duplicate in 2 parallel rows of VCs for lethal effects of the toxin (see plate design in Annex 4, Table 7). The penultimate dilution of the last positive response from this assay was then used as the starting dilution in a range of ten 1.3-fold dilutions. The aim was to report the results of 1 valid assay performed using the 2-fold dilution range and 3 valid assays performed using the 1.3-fold dilution range. However, the results from all of the assays performed, including invalid assays, were to be reported.

In vitro TCP assay

In vitro TCP assay was performed according to the methodology provided in Annex 5.

For each test toxoid, the indicative dilution steps provided in Annex 2 were used in a series of 9 dilutions made across a dilution plate (see plate design in Annex 5, Table 10). To each of the 9 dilutions was added an equal volume of the standard antitoxin (VI) diluted to a concentration of 0.8 IU/mL. A series of seven 1.1-fold dilution steps of CSTx2, centred on the TNE+ value of CSTx2, in volumes equal to that of the toxoid-antitoxin mixture was then added down the dilution plate. In addition, the same series of 7 dilutions of CSTx2 was added to a control column and each was mixed with the standard antitoxin at 0.4 IU/mL without the toxoid present. This acted as a check on the TNE+ value of the CSTx2 in each culture plate.

Each of the final mixtures was assessed on VCs for lethal effects of the CSTx2. The aim was to report the results of 1 valid assay performed using the 20-increment step dilution series and 3 valid assays performed using the 10-increment step dilution series. However, the results from all of the assays performed, including invalid assays, were to be reported.

5. DATA REPORTING, CALCULATIONS AND STATISTICAL METHODS

5.1. Data reporting

Participants were requested to report their results in the Excel data sheets provided by the study organisers. One separate sheet was provided for each step from I–III and 2 sheets

were provided for Step IV. Calculation formulas corresponding to the standardised calculation methods selected by the management team were included in the Excel sheets in order to allow the participants to report raw data only while immediately obtaining the information necessary to generate the calculated results (see section 5.2 for details).

Participants were requested to send the data obtained to the EDQM at the end of each step. This allowed the management team to evaluate the quality of the data obtained and to advise the participants regarding the need to either repeat assay(s) or move to the next step of the study.

5.2. Data processing and analysis

Only valid results were analysed; invalid results were not included in the calculation of the intra- and inter-laboratory precision. As a plate validity criterion for all of the *in vitro* assays (TNE+, MLD and TCP), the maximum coefficient of variation (CV) of the negative control OD values was set at 20 %. Above this CV the whole plate was considered invalid, and the assay had to be repeated.

Once this general plate validity criterion was met, further validity criteria for the end-points of the detector toxin and/or the test samples also had to be met in order for the results to be considered valid and processed further. For the specific end-point validity criteria for each assay, please refer to the corresponding SOPs in Annexes 3, 4 and 5.

5.2.1. Cell line sensitivity

In each assay, using the method described in the protocol, the participants assessed 6 series of ten 2-fold dilutions of CSTx2 (ranging from 1 in 8 000 to 1 in 4 096 000) in 1 plate to obtain 6 individual end-points. The final end-point (expressed as CSTx2 dilution factor) was the median of the 6 individual end-points.

5.2.2. Latent toxicity

In each assay, the participants produced a series of 2-fold dilutions (ranging from 1 in 5 to 1 in 2 560) for each of the test toxoids and a series of 2-fold dilutions (ranging from 5 to 0.009765625 IU/mL) for the standard antitoxin. Each series of dilutions was assessed, using the method described in the protocol, in 1 row on a VC plate to generate 1 individual end-point (expressed as inverse dilution) per test material.

5.2.3. In vitro TNE+

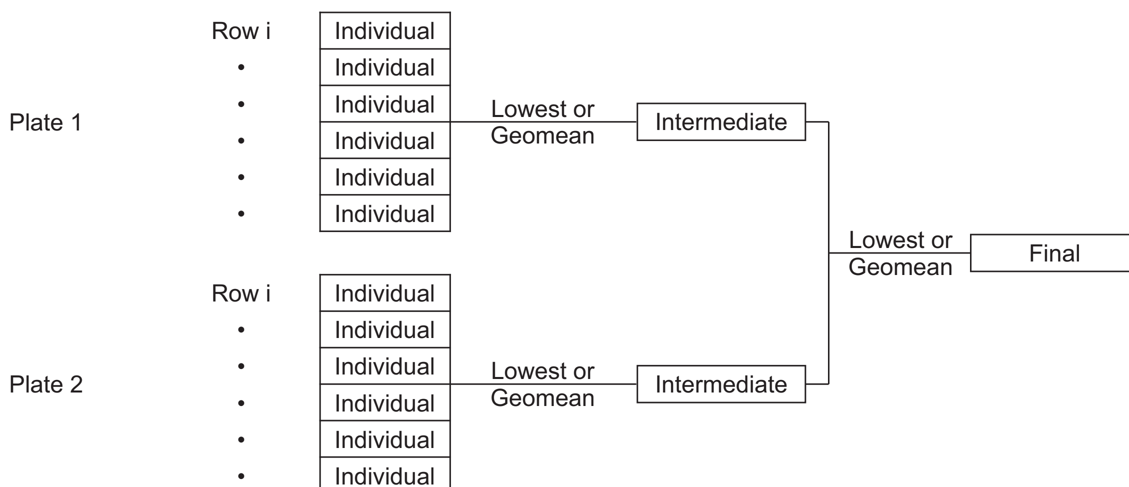
5.2.3.1. *IN VITRO* TNE+ OF THE DETECTOR TOXIN (CSTX2)

Each participant performed 1 initial assay using the method described in the protocol. For this, 6 series, each of ten 1.3-fold dilutions (ranging from 1 in 525 to 1 in 5 567), of the detector toxin (CSTx2) were produced. To each dilution was added an equal volume of standard antitoxin diluted to 0.2 IU/mL and the mixture was incubated to allow the toxin and antitoxin to react. Each series of reaction mixtures was assessed in 2 rows on a VC plate (Annex 4, Table 6). Two plates were required to accommodate all 6 series. Six individual end-points were generated on each plate. The intermediate end-points were the lowest individual end-points on each of the 2 plates. The final end-point (expressed in IU/mL) was the lowest of the 2 intermediate end-points, and it was used for the calculation of the starting dilution in the following assays.

Each participant then performed 3 independent replicate assays using the method described in the protocol. Each assay comprised 6 series each of ten 1.1-fold dilutions (where the starting dilution was the final end-point of the initial assay, described above, divided by 1.3) of the detector toxin (CSTx2). To each dilution was added an equal volume of standard antitoxin (VI) diluted to 0.2 IU/mL and the mixture was incubated to allow the toxin and antitoxin to react. Each series of reaction mixtures was assessed in 2 rows on a VC plate. Two plates were required to accommodate all 6 series. Six individual end-points were generated on each plate. The intermediate end-points were the GMs of the 6 individual end-points on each of the 2 plates. The final end-point was the GM of the 2 intermediate end-points. Three final end-points were generated

by the 3 independent replicate assays. Figure 1 is a flow diagram showing the generation of the different end-points in 1 of the assays.

Figure 1 – *Example of the generation of the different end-points in 1 of the assays (end-point: lowest value for the initial assay, GM for the 3 final assays)*



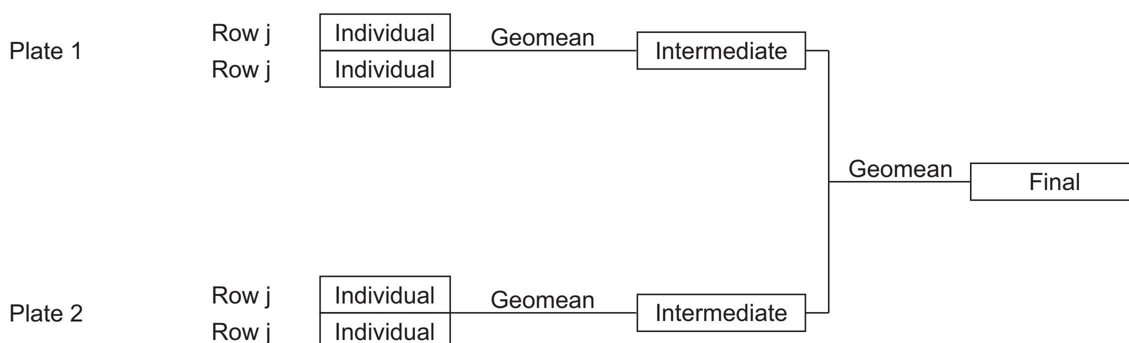
The TNE+ obtained by *in vitro* assays was a range determined as follows: the dilution factor of the toxin (not taking into account dilution by the addition of antitoxin) in each column was multiplied by 0.2 IU/mL (for further explanation see Annex 4). The column with the last positive well in a row marked the lower limit and the column with the first negative well in a row marked the upper limit. For the purpose of further calculations, the lower limit of this range was used as a point-estimate of the TNE+ value. If the lower limit differed by 1 dilution step in the parallel rows, the TNE+ value was calculated from the GM of the parallel end-point values.

5.2.3.2. IN VITRO TNE+ OF THE TEST TOXINS

Each participant performed 1 initial (range finding) assay for each test toxin, using the method described in the protocol. For each toxin a series of ten 2-fold dilutions was produced. To each dilution was added an equal volume of standard antitoxin diluted to 0.2 IU/mL and the mixture incubated to allow the toxin and antitoxin to react. Each series of reaction mixtures was assessed in 2 rows on each of 2 VC plates. Two individual end-points were generated on each of the 2 plates, giving a total of 4 individual end-points. The intermediate end-points were the GMs of the 2 individual end-points on each of the 2 plates. The final end-point was the GM of the 2 intermediate end-points.

Each participant then performed 3 replicate assays for each toxin, using the method described in the protocol. Each assay for each toxin comprised a series of ten 1.3-fold dilutions, where the starting dilution was the final end-point of the initial assay divided by 2. To each dilution was added an equal volume of standard antitoxin diluted to 0.2 IU/mL and the mixture incubated to allow the toxin and antitoxin to react. Each series of reaction mixtures was assessed in 2 rows on each of 2 VC plates. Two individual end-points were generated on each of the 2 plates, giving a total of 4 individual end-points. The intermediate end-points were the GMs of the 2 individual end-points on each of the 2 plates. The final end-point (expressed in IU/mL and calculated as described in 5.2.3.1) was the GM of the 2 intermediate end-points. Three final end-points were generated by the 3 replicate assays. Figure 2 is a flow diagram showing the generation of the different end-points in 1 of the assays.

Figure 2 – *Example of the generation of the different end-points in 1 of the assays*



5.2.4. *In vitro* TCP

Each participant performed 1 initial assay for each test toxoid, using the method described in the protocol. For each toxoid a series of nine 20-increment dilutions was produced and plated horizontally across the columns of a deep-well reaction plate. To each dilution was added an equal volume of standard antitoxin diluted to 0.8 IU/mL and in the wells of 1 of the unused columns was placed a set amount of standard antitoxin diluted to 0.4 IU/mL. The plate was incubated to allow the toxoid and antitoxin to react. A series of seven 1.1-fold dilutions of the detector toxin (CSTx2) was produced and each dilution was added to the wells across one row of the plate, including the column containing only the standard antitoxin, working down the plate. The plate was incubated to allow any antitoxin which was not bound to toxoid to react with the CSTx2. Each plate of reaction mixtures was assessed in 2 VC plates. The wells in the column containing only the antitoxin, at 1 concentration, and the CSTx2, at an increasing dilution, acted as a check on the TNE+ value of the detector toxin in each assay plate (see *In vitro* TNE+ method, Annex 4). Therefore, the end-point in this column was the well which most closely predicted the TNE+ value for the CSTx2 in this system. As the same concentration of CSTx2 was present in the wells across this row it was the end-point on this row which most accurately quantified the TCP value for the tested toxoid. Two individual end-points, 1 from each of the 2 VC plates, were therefore generated. The final end-point was the arithmetic mean (AM) of the 2 individual end-points. Each participant then performed 3 independent replicate assays for each toxoid, using the method described in the protocol. The method was essentially the same as that briefly described above, except that each toxoid was subjected to a series of nine 10-increment dilutions, where the starting dilution was the final end-point of the initial assay minus 40 TCP units, which corresponds to minus 2 dilution steps on the range finding plate. Two individual end-points, 1 from each of the 2 VC plates, were generated. The final end-point was the AM of the 2 individual end-points. Three final end-points were generated by the 3 independent replicate assays.

The TCP of *in vitro* assays was determined as follows: the end-point dilution for the TNE+ confirmation of the CSTx2 was used to identify the series of mixtures that contained the CSTx2 at the appropriate dilution. The dilution of the toxoid in the end-point of this series was used to calculate the TCP value of the toxoid. The TCP value was the end-point dilution value for the toxoid multiplied by 0.4 (for further explanation see Annex 5).

5.3. Statistical methods

A central statistical analysis of the results reported by the participants was performed by the EDQM. Repeatability and reproducibility of the methods was assessed by standard statistical techniques, such as analysis of variance, ranges and standard deviations. The occurrence of outliers and invalid assays was counted and compared by the use of contingency tables. A complete description of the statistical methods used in the analysis can be found in Annex 6.

6. RESULTS

All 14 laboratories reported results for cell line sensitivity, latent toxicity and TNE+ for detector toxin. Twelve of the laboratories also reported TNE+ values for the test toxins and TCP values for the toxoids.

6.1. Assessment of sensitivity of cell lines (Step I)

Fourteen laboratories reported the results of 18 assays in total. All the participants performed 1 assay with the exception of Laboratory 9 which tested 3 different VC lines and retained the values from the first cell line, which was used in all subsequent assays, and Laboratories 4 and 14 which reported the results of 2 valid assays, both of which were analysed. Laboratories 2 and 12 reported results from plates read at 2 different test wavelengths (570 nm and 630 nm). Only the results read at 570 nm were analysed.

Assay validity ratios. Out of the 18 assays reported, 17 were valid and taken into account in the central analysis. All the participants performed the assessment of the sensitivity of their respective cell line(s) and 13 (93 %) obtained valid assays at the first attempt.

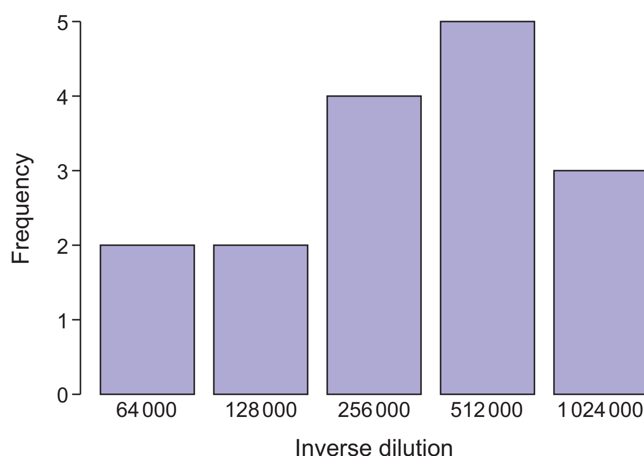
Of the 3 assays performed by Laboratory 9 with 3 different cell lines, one was invalid due to the positive results occurring in non-consecutive wells. The CV of the OD values recorded for the negative control wells in each assay ranged from 4 % to 17 % with the median at 8 %. For all assays, this was below 20 %, the maximum acceptable value which was set in the protocol.

ASSAY RESULTS

All individual end-points, including those which were not analysed (from invalid assays, OD readings at 630 nm and controls without cell lines) are shown in Annex 7. Analysis of the individual end-points showed them to be very homogeneous. In 12 of the 16 valid assays that were taken into account in the central analysis (75 %) the 6 individual end-points determined on each plate were identical. For the remaining 4 assays (25 %), a maximum difference of 1 dilution step among the 6 end-points per plate was observed.

The distribution of the final end-point values for the toxin sensitivities of the participants' VC lines (expressed as inverse dilution of the end-point) are displayed in Figure 3. Overall there was an up to 16-fold variation in the sensitivities of the different VC lines. The median final end-point value was 1 in 512 000, with 12 out of 16 (75 %) of the final end-points at this value or within ±1 dilution. The remaining 4 final end-points (25 %) were all within 3 doubling dilution steps below 1 in 512 000.

Figure 3 – *Frequency distribution of sensitivity final end-points*



6.2. Latent toxicity assessment of test materials (Step II)

Fourteen laboratories reported the results of 20 assays in total. All the participants performed 1 assay, with the exception of Laboratory 4 which reported the results of 1 invalid and 2 valid assays, Laboratories 10 and 14 which reported the results of 2 valid assays and Laboratory 13

which reported the results of 3 valid assays. Laboratories 2 and 12 reported results from plates read at 2 different test wavelengths (570 nm and 630 nm). Only the results read at 570 nm were analysed.

ASSAY VALIDITY RATIOS

Out of the 20 assays reported, 19 (95 %) were valid and taken into account in the central analysis. All the participants performed the latent toxicity assessment and 11 of them (79 %) obtained valid assays at the first attempt. Laboratory 4's first assay was invalid because the CV of the negative control wells was above 20 %; however, the second and third assays were valid. Of the 134 individual end-points obtained, only 14 (10 %) were invalid. In all cases, this was due to positive results not occurring in consecutive wells.

The CV of the OD values recorded for the negative control wells in each assay ranged between 4 % and 26 % with the median at 9 %. The only invalid CV value (26 %) was generated by Laboratory 4 in the first assay. The results of this assay were not included in the analysis. The distribution and median of the CV of the OD readings of the negative control wells in this step of the study were similar to those obtained in the sensitivity assessment of the VC lines (see Figure 7).

ASSAY RESULTS

All individual end-points are shown in Annex 8. Table 2 shows the frequency distribution of latent toxicity end-points and the median for each material.

In summary:

- **TdA** end-points were spread over 5 dilution steps (1 in 10 to 1 in 160) with a median of **1 in 40**.
- **TdC** end-points were spread over 4 dilution steps (1 in 20 to 1 in 160) with a median of **1 in 40**.
- **TdD** end-points were spread over 4 dilution steps (Low to 1 in 20) with a median of **1 in 5**.
- **TdN** end-points were spread over 2 dilution steps (Low and 1 in 5) with a median of **low**.
- **TdO** end-points were spread over 4 dilution steps (1 in 80 to 1 in 640) with a median of **1 in 320**.
- **TdP** end-points were spread over 6 dilution steps (Low and 1 in 10 to 1 in 160) with a median of **1 in 60**.

VI (Standard Antitoxin) end-points were all below the first dilution point. Therefore, the latent toxicity is low.

Table 2 – *Frequency distribution (number of observations) of latent toxicity end-points (expressed as inverse dilution of CSTx2)*

Material	Low	5	10	20	40	80	160	320	640	Median
TdA			4	3	7	1	2			40
TdC				3	6	7	1			40
TdD	3	9	3	2						5
TdN	15	2								Low
TdO						4	1	7	4	320
TdP	1		3	2	3	5	3			60
Antitoxin	19									Low

With the exception of 1 low value for TdP, the end-point values from all of the participants for each of the toxoids showed reasonably close grouping around the median. In the case of the standard antitoxin (VI), none of the participants found detectable latent toxicity in any of the assays.

6.3. Assessment of TNE+ value for detector toxin (Step III)

The 14 participants reported the results of 20 assays using the initial assay format (2 plates with a 1.3-fold dilution step). All the participants performed 1 assay with the exception of Laboratories 4, 11 and 14 which reported the results of 3 assays. Laboratory 2 reported results from plates read at 2 different test wavelengths (570 nm and 630 nm). Only the results read at 570 nm were analysed.

The 14 participants then reported the results of 50 assays using the final assay format (2 plates with a 1.1-fold dilution step). All the participants performed 3 assays with the exception of Laboratories 4, 6, 8 and 14 which reported the results of 4, 5, 4 and 7 assays, respectively. Laboratory 2 reported results from plates read at 2 different test wavelengths (570 nm and 630 nm). Only the results read at 570 nm were analysed.

ASSAY VALIDITY RATIOS

Out of the 70 (20 initial and 50 final) assays reported, 65 (93 %) were valid and taken into account in the central analysis. All the participants performed the assessment of the TNE+ value of CSTx2 and 9 of them (64 %) produced valid results for the 4 required assays at the first attempt. A total of 5 out of the 70 assays performed (7 %) were invalid. Laboratory 11 initially generated 2 sets of invalid results for the 1.3-fold assay, due to the CV of the negative control wells being above 20 %; Laboratory 14 had 1 invalid assay for the same reason. Laboratories 11 and 14 subsequently performed 1 and 2 valid 1.3-fold assays, respectively. The first 1.1-fold assays performed by Laboratories 4 and 8 were invalid due to high negative well CVs; however, both laboratories subsequently generated 3 valid 1.1-fold assays.

The CV of the OD readings of the negative control wells from the assays ranged between 2 % and 72 % with the median at 10 %. Five CVs above 20 %, the upper limit set in the protocol as validity criteria, were generated by Laboratories 4 (37 %), 8 (30 %), 11 (52 % and 72 %) and 14 (27 %). The results of the corresponding assays were not included in the central analysis. The distribution and median of the CV of the OD readings of the negative control wells in this step of the study were similar to those obtained in the sensitivity assessment of the VC lines and the latent toxicity assessment of the test materials (see Figure 7).

ASSAY RESULTS

All individual and intermediate end-points, including those resulting from invalid assays (which were not analysed) from the 1.3-fold dilution assays are shown in Annexes 9A and 9B.

For the initial 1.3-fold assay, the 6 individual end-points generated in each assay were identical in 76 % of plates, and differed by no more than 1 dilution step in 99 % of plates. The 2 subsequently calculated intermediate end-points per assay were identical in 72 % of the assays, and differed by no more than 1 dilution step in 98 % of assays. Table 3 shows the frequency distribution of the 37 intermediate end-points (in IU/mL, i.e. inverse dilution divided by 5) reported by the participants. The median value was 177 IU/mL, and 89 % of the end-points were at this value or within ± 1 dilution step. The distribution of final end-points, shown in Table 4, is similar to this, as the intermediate end-points differed by no more than one dilution step in 94 % of the assays.

Table 3 – *Frequency distribution of intermediate end-points for the CSTx2 TNE+ initial 1.3-fold dilution assays*

End-point (IU/mL)	Frequency	Percentage
105	1	37 %
177	19	51 %
231	13	35 %
300	4	11 %

Table 4 – *Frequency distribution of final end-points for the CSTx2 TNE+ initial 1.3-fold dilution assays*

End-point (IU/mL)	Frequency	Percentage
105	1	6 %
177	7	41 %
231	7	41 %
300	2	12 %

All of the intermediate end-points from the replicate 1.1-fold dilutions assays are available in Annexes 9C-E. Table 5 shows the distribution of the 92 intermediate end-points (in IU/mL). Successive classes differed by a 1.1-fold dilution step and started at 205 IU/mL. The end-points ranged from 215 to 439 IU/mL and were spread over eight 1.1-fold dilution steps (about 2.15-fold dilution range). In addition, end-points were between 226 and 330 IU/mL (1.5-fold ratio) in about 75 % of cases.

Table 5 – *Frequency distribution of intermediate end-points for the CSTx2 TNE+ replicate 1.1-fold dilution assays*

End-point (IU/mL)	Frequency	Percentage
[205-226]	4	4 %
[226-248]	20	22 %
[248-273]	16	17 %
[273-300]	11	12 %
[300-330]	21	23 %
[330-363]	7	8 %
[363-399]	9	10 %
[399-439]	4	4 %

Table 6 shows the final end-points together with the GMs and GCVs obtained by the participants. As an example, the 3 final end-points from Laboratory 1 had a GM of 234 IU/mL with a GCV of 8 %. The GCVs ranged from 0 % to 11 %, showing good repeatability of the test. On average, the repeatability was GCV = 6 %. The GMs of the participants' results ranged from 227 (Laboratory 7) to 407 IU/mL (Laboratory 9). Overall, the distribution was centred on 290 IU/mL, with a GCV of reproducibility of 20 %.

Table 6 – *Frequency distribution of intermediate end-points for the CSTx2 TNE+ replicate 1.1-fold dilution assays*

Laboratory	Rep. 1	Rep. 2	Rep. 3	GM	GCV
1	252	215	237	234	8
2	315	315	315	315	0
3	287	261	267	271	5
4	248	239	261	249	5
5	230	253	230	237	6
6*	237	235-261	228	242	6
7	231	235	215	227	5
8	278	297	306	293	5
9	439	363	422	407	11
10	330	327	325	327	1
11	348	379	370	366	5
12	334	329	299	320	6
13	371	368	354	365	3
14†	287	261	261	269	6
14	306	278	306	297	6
			Overall	290	20

* Laboratory 6 obtained an unexpectedly low OD in a single well on 1 plate during the second replicate of the assay, requiring an extra assay to be carried out. This isolated OD value was ignored as part of the analysis, resulting in 4 homogeneous final end-points (CV = 6 %).
† Laboratory 14 reported the results of 2 series of 3 independent replicate assays.

The histogram below (Figure 4) shows the distribution of the final end-points obtained by the participants in the 1.1-fold dilution assay.

Figure 4 – *Frequency distribution of final end-points (IU/mL) for the CSTx2 TNE+ (replicate 1.1-fold dilution assays)*

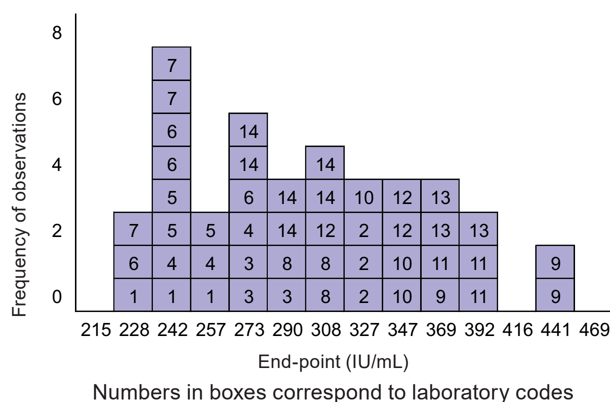
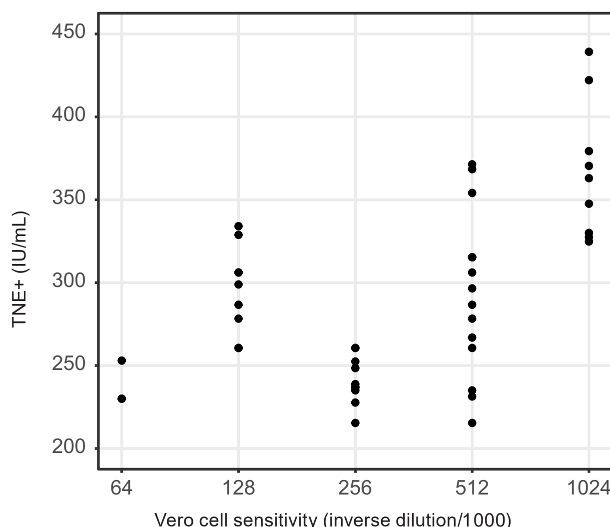


Figure 5 shows the distribution of the final end-points as a function of the sensitivity of the different VC lines to CSTx2. Significantly higher end-points were obtained with VCs with a sensitivity of 1 in 1 024 000 (see Annex 7 and Figure 6 for the individual assay results for the sensitivity of VC lines to CSTx2 expressed as inverse dilution/1 000).

Figure 5 – *CSTx2 TNE+ values versus sensitivity of VC lines to CSTx2*



6.4. Assessment of test materials (Step IV)

6.4.1. TNE+ values of Toxins

The 14 participants reported the results of 16 assays using the initial assay format (toxins tested using a 2-fold dilution step). All the participants performed 1 assay, with the exception of Laboratories 6 and 8 which reported the results of 2 assays. Laboratory 2 reported results from plates read at 2 different test wavelengths (570 nm and 630 nm). Only the results read at 570 nm were analysed.

Thirteen participants reported the results of 46 assays using the final assay format (toxins tested using a 1.3-fold dilution step). Laboratory 14 did not report any results. All remaining participants performed 3 assays, with the exception of Laboratories 7, 8, 9 and 11 which reported the results of 7, 4, 4 and 4 assays, respectively. Laboratory 2 reported results from plates read at 2 different test wavelengths (570 nm and 630 nm). Only the results read at 570 nm were analysed.

ASSAY VALIDITY RATIOS

Out of the 62 (16 initial and 46 final) assays reported, 53 (85 %) were valid and taken into account in the central analysis. Thirteen of the 14 participants (93 %) performed the TNE+ assessment of the test toxins and 8 of them (57 %) produced valid results for all 4 required assays at the first attempt. Nine out of the 62 assays performed (14.5 %) were invalid. Four invalid assays were due to the CV of the negative control wells being above 20 % (Laboratories 7 and 8: 1 assay each and Laboratory 13: 2 assays) and 5 were due to there being more than 2 dilution steps between the calculated and expected CSTx2 end-points (Laboratories 6 and 11: 1 assay each and Laboratory 7: 3 assays). All of these participants subsequently performed the requisite number of valid 1.3-fold assays, with the exception of Laboratory 13 who submitted only 1 valid 1.3-fold assay result for 2 of the toxins.

The CV values of the OD readings of the negative control wells from the assays ranged between 1 % and 25 %, with the median at 9 %. The 5 invalid high CVs were generated by Laboratories 7 (23 %), 8 (25 %) and 13 (21 %, 22 % and 24 %). The results of these assays were not included in the analysis. The distribution and median of the CV of the OD readings of the negative control wells in this step of the study were similar to those obtained in the sensitivity assessment of the VC lines, the latent toxicity testing and the TNE+ evaluation of CSTx2 (see Figure 7).

ASSAY RESULTS

The detector toxin CSTx2 was included as a control toxin for assessment in all of the TNE+ assays and occupied 2 rows on each VC plate. Examination of all of the individual end-points

obtained for CSTx2 revealed that they were identical in 87 % of plates, differed by no more than 1 dilution step in 99 % of the plates and by no more than 2 dilution steps in all the plates. The overall mean TNE+ value for CSTx2 calculated from the end-points generated in this step of the study was 293 IU/mL, which confirms remarkably well the value of 290 IU/mL obtained in Step III of the study (see Table 6).

Analysis of the individual end-points for each toxin showed that both end-points for a toxin were identical in 91 % of plates, and differed by no more than 1 dilution step in all the plates. Similar analysis of the intermediate end-points showed that both end-points for a toxin were identical in 90 % of assays, differed by no more than 1 dilution step in 99 % of the plates and by no more than 2 dilution steps in all the plates.

Table 7 shows the final end-points from the 2-fold dilution assays, in IU/mL, reported by the participants for each toxin. The participants' end-points are homogeneous as they do not differ from the GM or median values by more than one dilution step for any given toxin.

Table 7 – *Final end-points for the TNE+ initial assays of toxins (expressed in IU/mL)*

Laboratory	TxR	TxS	TxV	TxW	TxY	TxZ
1	8	16	16	128	128	64
2	16	32	16	128	256	64
3	16	32	16	128	256	64
4	16	32	32	128	256	64
5	16	23	32	91	128	64
6	8	16	16	128	128	64
7	16	32	32	128	256	64
8*	23-32	32-32	32-32	256-256	256-512	128-128
9	16	32	32	128	256	91
10	16	32	32	181	256	64
11	32	32	32	128	256	128
12	16	32	16	128	256	64
13	16	32	32	128	256	64
14	16	32	32	128	256	64
GM	16	29	25	140	233	75
Median	16	32	32	128	256	64

*Laboratory 8 reported the results of 2 valid assays.

All of the final end-points obtained by the participants for the 6 toxins in the replicate 1.3-fold dilution assays are detailed in Annex 10. Table 8 summarises the data, showing the overall GMs and CVs calculated for the participants' results.

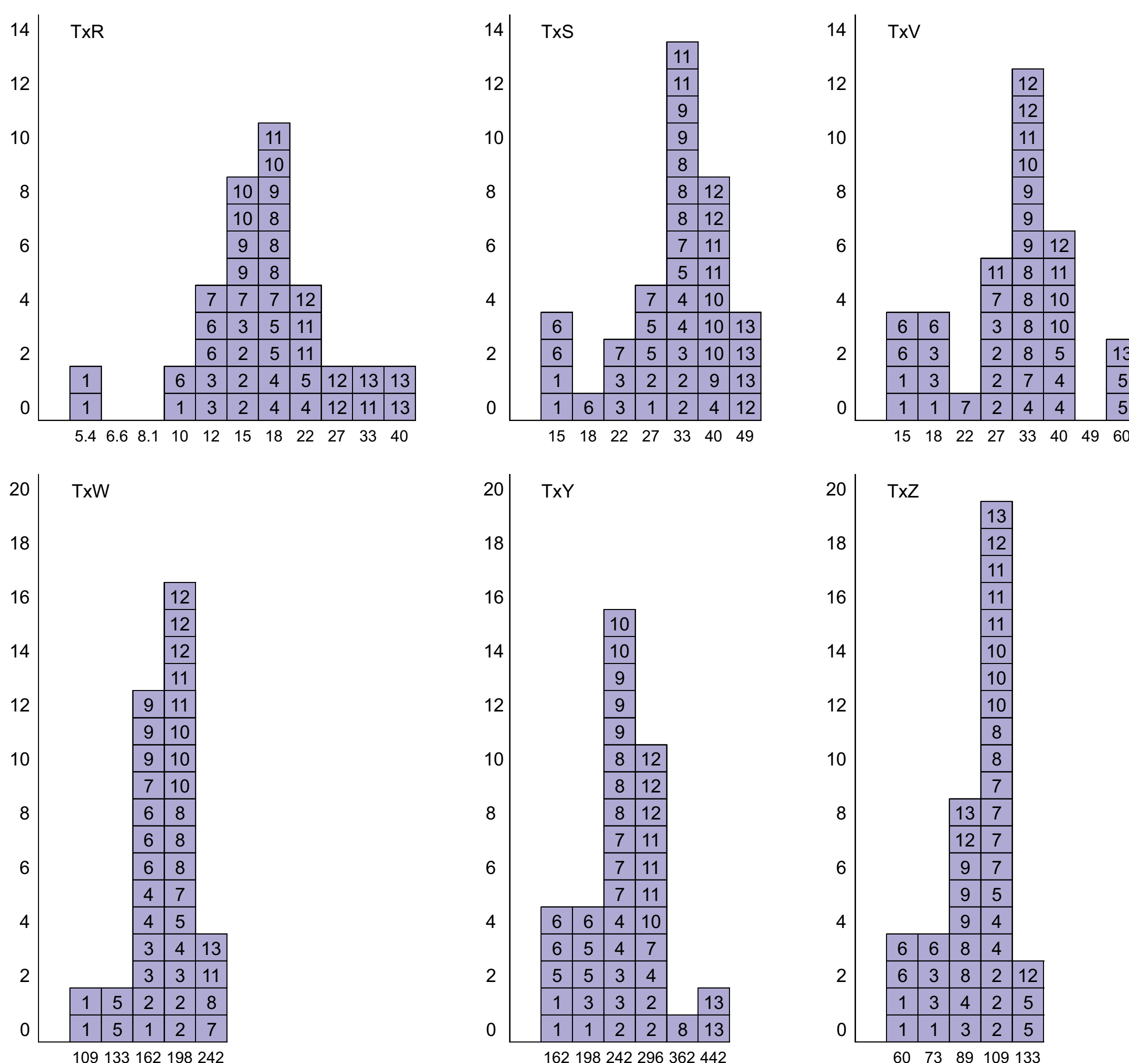
The repeatability of the test, i.e. the variability between the final end-points of the replicate assays, ranged from 11 % (TxZ) to 20 % (TxR). These figures were averaged over the participants' repeatability estimates, which ranged, for example, from GCVs of 0 % (Laboratories 2, 7, 9, 10 and 11) to 22 % (Laboratory 12) for TxZ (see Annex 10). The reproducibility of the test, i.e. the variability between the GMs of the participants, ranged from 20 % (TxW) to 51 % (TxR). The greater variability observed for toxins TxR, TxS and TxV (37 % to 51 %) could be due to the low specific toxin concentrations in the samples being close to the limit of quantification of the test. For the test toxins with higher toxin concentrations, the repeatability and reproducibility of the test were closer to 15 % and 30 %, respectively. It should be noted that TxY and TxZ were obtained by dilution of the detector toxin CSTx2 (1.2-fold and 3-fold dilution, respectively). The overall GM values of 225 and 85 IU/mL for these 2 toxins were reasonably close to theoretical values predicted from the dilution factors, i.e. 242 and 97 IU/mL, respectively.

Table 8 – *Summary statistics of the TNE+ replicate 1.3-fold dilution assay of toxins*

Toxin	Overall GM (IU/mL)	GCV Repeatability	GCV Reproducibility
TxR	15	20	51
TxS	27	16	37
TxV	27	15	49
TxW	165	13	20
TxY	225	14	28
TxZ	85	11	21

Figure 6 shows the distribution of the final end-points obtained for the 6 test toxins, where the figures within the squares are the laboratory codes. Successive mid-points of classes (x-axis) differ by the same fold ratio, allowing comparison of the spread of the distributions regardless of the mean values of the toxins. It is noticeable that the test toxins with lower specific toxin concentrations (TxR, TxS and TxV) yielded more variable TNE+ value results than the test toxins with the high specific toxin concentrations (TxW, TxY and TxZ). (NB The numerical differences in the end-points cannot be directly translated to the differences in TNE+ values as the latter is calculated from the end-points by multiplying them by 0.2 (see Annex 4. “Data Interpretation”, point *c*, page 142). Therefore, a 100 difference between two end-points is translated to a 20 unit difference in the TNE+ values.)

Figure 6 – *TNE+ final end-points (IU/mL) obtained for the 6 test toxins (figures within the squares are the laboratory codes)*



6.4.2. TCP values of Toxoids

The 14 participants reported the results of 41 assays using the initial assay format (toxoids tested using 20-increment dilutions). All the participants had to perform several assays to obtain 1 valid assay, with the exception of Laboratories 5, 6 and 13, which reported valid results at the first assay.

Eleven participants reported the results of 51 assays using the final assay format (toxoids tested using 10-increment dilutions). Laboratories 1, 13 and 14 did not report any results. The participants had to perform more than 3 assays to obtain 3 valid results for the various toxoids, with the exception of Laboratories 5 and 10 which reported valid results for all the toxoids in 3 consecutive assays. Laboratory 7 reported the results of 2 assays only.

ASSAY VALIDITY RATIOS

Out of the 92 (41 initial and 51 final) assays reported, 76 (83 %) were valid and taken into account in the central analysis. Nine of the 11 participants (82 %) performed the TCP assessment of all of the test toxoids and 2 (18 %) performed the assessment of some of the toxoids. Only 1 of the participants (9 %) produced 4 (1 initial + 3 final) valid required assays at the first attempt. A total of 16 out of the 92 assays performed (17 %) were invalid. Of these, 3 were invalid due to the CV of the negative control wells being above 20 %, 3 due to the CSTx2 end-point being more than 2 dilution steps from the target value, 1 due to CSTx2 being positive in non-consecutive wells and 9 due to the CSTx2 end-point being out of range.

A total of 89 out of the 631 TCP end-points obtained (14 %) were invalid. Table 9 shows that, in most cases, invalid end-points resulted from wells that were all positive or negative (end-point out of the tested dilution range). Toxoids TdA, TdO and TdP were particularly affected.

Table 9 – *Sources of invalid TCP end-points for toxoids*

Source	TdA	TdC	TdD	TdN	TdO	TdP	Total
Non-consecutive positive wells	3	1		1	5	4	14
End-point out of range	23	3			30	19	75

In practice, most of the participants adjusted the dilutions used in the first well to obtain quantified end-points (Table 10). For example, while the recommended dilution in the first well for toxoid TdA was 1 in 40, Laboratories 1 and 7 used 1 in 80, Laboratory 4 used 1 in 100, Laboratories 10 and 11 used 1 in 120, Laboratories 7 and 9 used 1 in 140, and Laboratory 12 used 1 in 160, 1 in 180 and 1 in 200.

Table 10 – *Inverse of first well dilutions used for initial TCP assays*

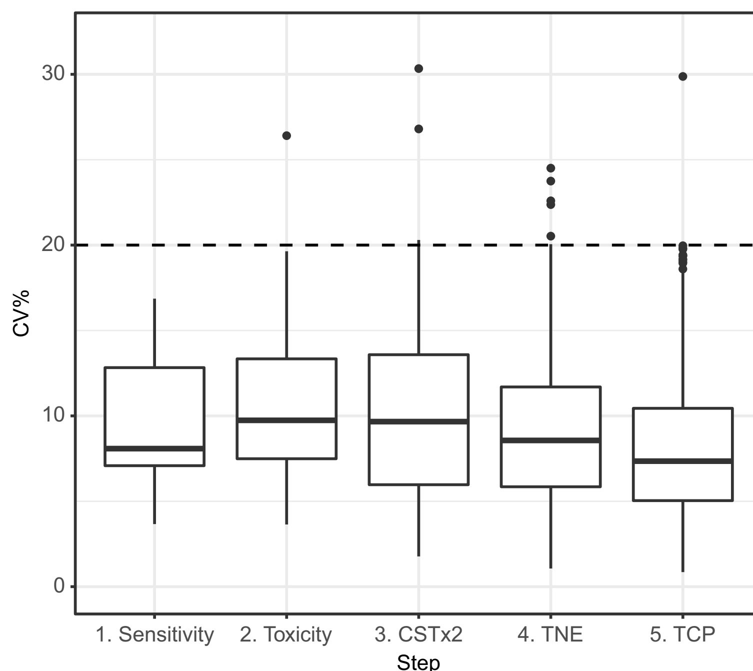
Toxoid	Inverse First Well Dilutions (Laboratories)
TdA	**40,** 80 (1, 7), 100 (4), 120 (10, 11), 140 (7, 9), 160 (12), 180 (12), 200 (12)
TdC	**10** (all laboratories)
TdD	**20** (all laboratories)
TdN	**20** (all laboratories)
TdO	**100,** 140 (4, 7), 160 (1), 180 (8, 11), 200 (1, 2, 9, 12), 220 (7, 10, 12)
TdP	**140,** 160 (4), 200 (1, 9), 220 (7, 8), 240 (10)

Numbers in **bold** refer to the inverse of the recommended dilution in the first well.

The boxplot in Figure 7 shows the CV of the OD readings of the negative control wells from the assays, together with the same boxplots from the cell line sensitivity, latent toxicity, CSTx2 TNE+ assessments and TNE+ values of toxins, for comparison. The CV values ranged between 1 % and 104 % with the median at 8 %. The 4 invalid assays linked to high CVs were generated by Laboratories 7 (30 %), 9 (103 % and 104 %) and 12 (41 %). The results of these assays were

not included in the analysis. The distribution and median of the CVs of the OD readings of the negative control wells in this step of the study were similar to those obtained in the earlier parts of the study.

Figure 7 – *Coefficients of variation (CVs) of the OD readings of the negative control wells. (The validity threshold used in the study is identified by the dotted line; CVs above 30 % are not displayed)*



ASSAY RESULTS

Examination of all of the individual end-points obtained for the TNE+ value of CSTx2 in this step of the study revealed that the end-points were identical in 99 % of cases and differed by no more than 1 dilution step in all the plates. The overall mean TNE+ value for CSTx2 calculated from the 639 end-points generated in this step of the study was 321 IU/mL. This is higher than the value of 290 IU/mL obtained in Step III of the study (see Table 6).

Analysis of the individual TCP end-points for each toxoid showed that the end-points were identical in 77 % of cases, differed by 1 dilution step in 95 % of cases and by no more than 2 dilution steps in all cases.

Table 11 shows the final end-points from the initial 20-increment dilution assays, in TCP units, reported by the participants for each toxoid, together with the AMs and medians.

Table 11 – *Final end-points for initial 20-increment dilution TCP assays of toxoids (expressed in TCP units)*

Laboratory	TdA	TdC	TdD	TdN	TdO	TdP
1	72	8	32	8	–	136
2	56	4	24	8	128	88
3	72	4	24	16	88	84
4	60	10	24	16	104	88
5	64	26	56	44	80	96
6	72	4	24	8	96	72
7	–	–	32	16	–	–
8	64	8	32	16	124	120
9	88	8	40	16	136	120
10	80	4	24	16	140	124
11	60	10	32	16	96	96
12	96	4	24	16	104	112
13	27	–	–	–	–	–
14	16	4	8	8	40	56
AM	64	8	29	16	103	99
Median	64	6	24	16	104	96

All the final end-points obtained by the participants for the 6 toxoids in the replicate 10-increment dilution assays are shown in Annex 11, together with the AMs and CVs. Table 12 summarises the data, showing the overall AMs and CVs calculated for the participants' results. (NB The numerical differences in the end-points cannot be directly translated to the differences in TCP values as the latter is calculated from the end-points by multiplying them by 0.4 (see Annex 5. "Data interpretation", point *c*, page 146). Therefore, a 100 difference between two end-points is translated to a 40 unit difference in the TCP values.)

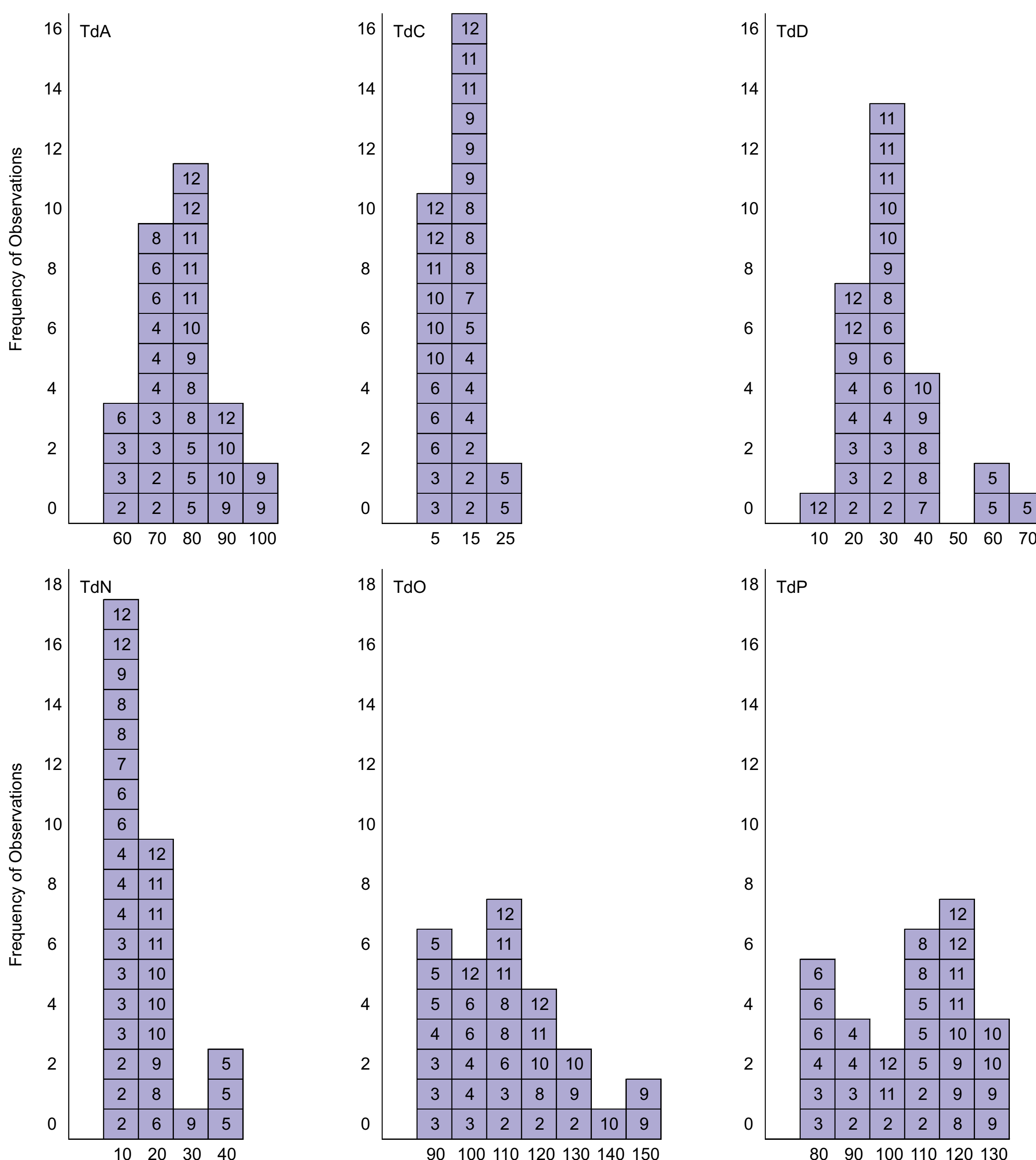
The repeatability of the test, i.e. the variability between the final end-points of the replicate assays, ranged from 7 % (TdA, TdO and TdP) to 21-22 % (TdC, TdD and TdN). These figures were averaged over the participants' repeatability estimates, which ranged, for example, from CVs of 0 % (Laboratory 5) to 11 % (Laboratory 2) for TdA (see Annex 11). The reproducibility of the test, i.e. the variability between the AMs of the participants, ranged from 13 % (TdA) to 52 % (TdN). Greater variability in reproducibility was observed for toxoids TdC, TdD and TdN (39 % to 52 %) which had lower TCP values. For the toxoids with higher TCP values, the repeatability and reproducibility of the test were no higher than 7 % and 15 %, respectively.

Table 12 – *Summary statistics of replicate 10-increment dilution TCP assays of toxoids*

Toxoid	Overall AM (TCP units)	CV Repeatability	CV Reproducibility
TdA	76	7	13
TdC	7	21	51
TdD	32	22	39
TdN	17	22	52
TdO	112	7	15
TdP	105	7	15

Figure 8 shows the distribution of the final TCP end-points of the 6 toxoids, where the figures within the squares are the laboratory codes. Successive mid-points of classes (x-axis) differ by the same number of TCP units, allowing comparison of the spread of the distributions whatever the mean values of toxoids.

Figure 8 – *Histograms of final TCP end-points of the 6 test toxoids (expressed in TCP units) (Figures within the squares are the laboratory codes)*



7. DISCUSSION AND CONCLUSIONS

7.1. Participants' VC lines: suitability, sensitivity and comparison between laboratories (Step I)

The ODs of the VC negative control wells in all steps of this study showed similar ranges of CVs with very similar medians. This suggests that all of the participants' VC lines produced similar consistent confluent monolayers in almost all of the wells of the flat-bottomed tissue culture plates used in all parts of the study. This, in turn, means that the basic conditions at the start of all of the assays in all of the participants' laboratories were similar and that the outcomes of the assays are, therefore, expected to be comparable. The VC lines used in the 14 laboratories participating in this study showed a 16-fold range of sensitivity to *C. septicum* toxin used as

the detector toxin (CSTx2). The maximum level of toxin sensitivity varied from a dilution of 1 in 64 000 (cell lines of Laboratories 5 and 14) to 1 in 1 024 000 (cell lines of Laboratories 9, 10 and 11; see Figure 3). This is similar to the range of maximum sensitivities, of 1 in 23 000 to 1 in 562 000, found with the VC lines used in Part 1 of the study [18], although the detector toxin was different in that study. In addition, the median sensitivity of 1 in 512 000 in this study is similar to the median of 1 in 300 000 in Part 1 of the study [18]. This suggests that all the VC lines used by the participants were suitably sensitive to *C. septicum* toxin and that results obtained in the present study are in line with the expectations raised by Part 1 of project BSP130.

7.2. Latent toxicity of the standard antitoxin (VI) and of test toxoids (Step II)

The lack of detectable latent toxicity for the standard antitoxin (VI), at 5 IU/mL, coincides with the results obtained for VI in Part 1 of BSP130 and confirms that the presence of the standard antitoxin in the final TNE+ and TCP mixtures that were applied to the VCs would not have had any interfering effect on the assay outcomes. In contrast, almost every participant detected some level of latent toxicity associated with all of the test toxoids. This was not unexpected as, although all of the toxoids had passed residual toxicity testing in mice, the VC assays are, on average, 1000-fold more sensitive than the *in vivo* tests [18]. The only exception was TdN, where only 1 laboratory found some latent toxicity, and that only at the highest concentration (1 in 5).

7.3. Assessment of the activity of the detector toxin (CSTx2) (Step III and Step IV)

The activity of the detector toxin (CSTx2) was assessed by all laboratories through a dedicated TNE+ test, and all laboratories reported values. The corresponding repeatability was good in all the participant laboratories with between assay GCVs ranging from 0 % to 11 % with an average of 6 %.

The respective GM activity values obtained by the participants for CSTx2 ranged from 227 (Laboratory 7) to 407 IU/mL (Laboratory 9). Overall, the distribution was centred on 290 IU/mL, with an acceptable reproducibility (GCV) of 20 %.

The TNE+ assessment of CSTx2 activity in the context of the TNE+ assessment of test toxins in Step IV also generated several interesting findings. The overall mean TNE+ value generated for CSTx2, which was included on all the assay plates in Step IV of the study for comparison, was 293 IU/mL. This matches very well with the value of 290 IU/mL obtained in the actual TNE+ assessment of the detector toxin in Step III of this study.

As part of the TCP assay of the test toxoids, the TNE+ value of CSTx2 was assessed – once again – in each test run. The overall mean TNE+ value for CSTx2 calculated from all the end-points generated in the TCP assays in Step IV of the study was 321 IU/mL. This is higher than the values of 290 IU/mL obtained in Step III of the study and 293 IU/mL obtained in the TNE+ assessment of the test toxins in Step IV. This difference may have been caused by the different assay design (e.g. slightly different dilution schemes and different assay volumes).

7.4. Assessment of the activity of the test toxins by the TNE+ assays (Step IV)

In the initial 2-fold dilution range finding assay, the end-points (IU/mL) reported by the participants for each toxin were homogeneous; they did not differ from the GMs or median values by more than 1 dilution step, thus demonstrating the good reproducibility of the TNE+ assay at this level of dilution series.

In the replicate 1.3-fold dilution TNE+ assays the repeatability of the assay, i.e. the variability between the final end-points of the replicate assays, ranged from 11 % (TxZ) to 20 % (TxR). These figures were averaged over the participants' repeatability estimates, which ranged, for example, from GCV = 0 % (Laboratory 2) to 16 % (Laboratory 1) for TxZ (see Annex 9). The reproducibility of the test, i.e. the variability between the GMs obtained by the participants for

each toxin, ranged from 20 % (TxW) to 51 % (TxR). The greater variability in reproducibility observed for toxins TxR, TxS and TxV (37 % to 51%) is probably due to the fact that the specific toxic activity of these toxins is close to the limit of quantitation. This could therefore result in a decrease in the reproducibility observed for the TNE+ values obtained for these toxins. For the test toxins with higher specific toxic activity (TxW, TxY and TxZ) the repeatability and reproducibility of the TNE+ test were 15 % and 30 %, respectively, which is acceptable.

The good precision and linearity of the 1.3-fold dilution TNE+ assays for materials containing relatively high concentrations of specific toxin is demonstrated by the values generated for TxY and TxZ. Toxins TxY and TxZ were prepared by 1.2-fold and 3-fold dilution of the detector toxin CSTx2 (TNE+ value 290 IU/mL, as calculated in Step III of this study), respectively. The overall GM values of 225 IU/mL and 85 IU/mL for these 2 toxins are reasonably close to the values predicted from the dilution factors, i.e. 242 IU/mL and 97 IU/mL.

7.5. Assessment of the activity of the test toxoids (Step IV)

For the replicate 10-increment dilution TCP final assays, the repeatability of the assay, i.e. the variability between the final end-points of the assays, ranged from 7 % (TdA, TdO and TdP) to 21–22 % (TdC, TdD and TdN). These figures correspond to the average of the individual repeatability estimates obtained by the participants, which ranged, for example, from CVs of 0 % (Laboratory 5) to 11 % (Laboratory 2) for TdA (see Annex 11). The reproducibility of the test, i.e. the variability between the AM obtained by the participants for each toxoid, ranged from 13 % (TdA) to 52 % (TdN). The greater variability in reproducibility observed for toxoids TdC, TdD and TdN (39 % to 52 %) is probably due to the fact that the low TCP values of these toxoids are close to the limit of quantitation. For the toxoids with higher TCP values (TdA, TdO and TdP) the repeatability and reproducibility of the test were very satisfactory at 7 % and 15 % respectively.

7.6. Comparison of the results obtained in the 2 parts of BSP130

When compared with the equivalent *in vivo* and *in vitro* tests performed as part of the first collaborative study (BSP130 Part 1) [18], the repeatability and reproducibility of the optimised assays used in this study (BSP130 Part 2) were, in almost all cases, much improved.

7.6.1. TNE+ in Part 2 versus in vivo testing and in vitro MLD in Part 1

The newly introduced TNE+ assay used here for the assessment of toxicity of test toxins is the *in vitro* equivalent of the L+ *in vivo* tests that can be used for the assessment of toxicity in *C. septicum* vaccine control. In Part 2 of the study, TNE+ was used instead of the MLD that was used in Part 1. Due to high dependency on the sensitivity of the VC line used, normalised results could not be obtained for the *in vitro* MLD procedure in Part 1 (thus the results of experiments performed in different laboratories could not be compared).
Whereas the *in vivo* MLD test in mice had GCVs for repeatability and reproducibility of 25 % to 47 % and 69 % to 117 %, respectively, the GCVs for repeatability and reproducibility for the TNE+ assay in this study were 11 % to 20 % and 20 % to 51 %, respectively. These values are also better than those obtained for the *in vitro* MLD test in VCs performed in Part 1 of the study, which were 24 % to 50 % and 43 % to 77 % for repeatability and reproducibility, respectively.

7.6.2. Optimised in vitro TCP assay in Part 2 versus in vivo and in vitro TCP assays in Part 1

In Part 2 of the study, the CVs for repeatability and reproducibility of the TCP were 7 % to 22 % and 13 % to 52 %, respectively. These are better than those observed in Part 1 for *in vitro* TCP test values (repeatability: 17 % to 35 % and reproducibility: 23 % to 51 %) and for the TCP *in vivo* test values (repeatability 0 % to 31 % and reproducibility 31 % to 110 %).

7.6.3. Percentage of invalid assays

The percentage of invalid assays observed in Part 2 was higher than in Part 1. It is postulated that this observation could be linked to the increase in complexity of the assay procedures between the 2 parts of the study.

It is also noteworthy that up to 5 % of assays were invalid due to the CVs of the negative control wells on the plate being above the limit of 20 % that was set in the procedures provided in the study protocol. In all cases where assays were invalid due to the negative control CVs being too high, the calculated end-points would have been equal to or very close to those of subsequent valid assays (see Annexes 8 and 9A-E). Based on this observation, it is recommended to set the assay validity criteria for the maximum CV of the negative control wells on the plates at 25 % for routine QC laboratory testing.

Although all but one of the participants were unfamiliar with the complex TNE+ and TCP assays, the 13 participants that performed the TNE+ evaluations of the test toxins and the 11 participants that performed the TCP assessments of the test toxoids were ultimately all able to obtain replicate results with acceptably low levels of invalid assays (16 %) for both assay procedures.

7.7. Suitability of the proposed methods for the purpose of routine testing of toxin and toxoid batches at veterinary vaccine manufacturers' quality control laboratories

The intra-laboratory precision observed was in general satisfactory, both for the toxin (TNE+) and toxoid (TCP) *in vitro* assays in Part 2. Although the results obtained for materials with high concentrations showed better reproducibility than those with low concentrations, the methods allowed clear discrimination between low and high concentration bulks. These observations are important as toxin bulks with low levels of toxicity are unlikely to be properly toxoided and processed. Similarly, toxoid bulks with low antigenicity are unlikely to be included in final vaccines as they would occupy too large a volume for a multicomponent vaccine. Therefore it can be assumed that TNE+ and TCP assays will enable manufacturers to determine whether toxin and toxoid bulks are of sufficient concentration to be worth processing. More importantly, the accurate assessment of toxoids with high TCP values will allow manufacturers to reproducibly formulate final vaccines which contain sufficient toxoid to be potent enough but without wasting excess antigen or risking unacceptably high levels of antigenic competition.

7.8. Recommendations for the implementation of the *in vitro* TNE+, MLD and TCP tests for use in routine QC

Some adaptations of the assay procedures used in Part 2 of BSP130 should be envisaged in order to allow their use in routine in-process toxicity and antigenicity testing of *C. septicum* vaccines. These adaptations should make it possible to reduce the number of invalid assays and increase the throughput of test samples. Firstly, it is recommended that the validity criterion for the CV of the negative control wells should be set to 25 % for all the VC assays (see section 7.6). Secondly, once the laboratory has performed the necessary in-house validation steps and become familiar with the assays, and has determined that the level of precision reached is acceptable, it may be possible to simplify the assay design. Examination of assay results showed that the OD values from duplicate rows and plates were very consistent. This finding together with the good repeatability CVs of the assays suggests that once a laboratory is sufficiently proficient, the duplicate testing of samples may be omitted. This would result in significant savings in resources, materials and time.

7.9. Summary of conclusions

The *in vitro* TNE+ and TCP VC line-based assays developed and characterised in the second part of the BSP130 project represent an optimisation of the *in vitro* assays assessed previously in Part 1 [18] and provide repeatable and reproducible results, which are far more accurate and precise than those obtained with the corresponding *in vivo* MLD and TCP mouse tests.
These methods are relatively easy to transfer to QC laboratories which, even though unfamiliar with the assay methods, seem to rapidly master them as demonstrated by the relatively low levels of invalid assays.

In addition, analysis of the results of this study suggested that simplifying the design of these assays, through a few minor modifications (relaxation of validity criteria and reduction of

duplicate and repeat testing), could be easily achieved while maintaining the desired accuracy and precision.

As they allow accurate and quantitative measurement of the respective toxicity and antigenicity of bulk toxins and toxoids, the *in vitro* TNE+ and TCP VC line-based assays are fit for the purpose of controlling *C. septicum* veterinary vaccines in routine in-process quality controls. Therefore these tests should facilitate phasing out of the currently used *in vivo* tests and have the potential to become compendial tests. It is also postulated that the implementation of these techniques in routine QC could generate significant improvements in the formulation of the *C. septicum* component of clostridial multicomponent vaccines while allowing significant savings in time and resources.

8. PERSPECTIVES

The results obtained in Part 1 [18] and in the present study were disseminated through the study sponsors, i.e. the EPAA [16] and the EDQM [17] and at international workshops (Egmond aan Zee, Netherlands, 15-16 September 2015; 10th World Congress on Alternatives and Animal Use in the Life Sciences Seattle, Washington, USA September 2017).

In parallel, the project preparatory phases, as well as Part 1 and Part 2 outcomes, were regularly presented to the EPAA steering committee, the BSP steering committee and to Group of Experts 15V of the Ph. Eur. The latter groups considered that the *in vitro* methods established in BSP130 could represent valuable alternatives to the *in vivo* MLD and TCP currently used in clostridial vaccine in-process controls. Beside the conclusions based on the observations of the study (detailed in section 7), the possibility that similar optimised *in vitro* assays could potentially be used for all cytotoxin-based clostridial antigens was also considered.

As a consequence, the Ph. Eur. Commission mandated Group 15V to revise the monographs on *Clostridium novyi (type B) vaccine for veterinary use (0362)*, *Clostridium perfringens vaccine for veterinary use (0363)* and *Clostridium septicum vaccine for veterinary use (0364)*. The final drafts of the revised monographs [21–23] were submitted to a public enquiry. Laboratories involved in testing these products were strongly encouraged to take part in the public enquiry and to use this study as a basis for developing and implementing their own tests.

To monitor the current field situation, a consultation was held with all interested parties (Ph. Eur. experts, regulators, control laboratories, manufacturers, NGOs and others) to share experience and provide guidance on the development and application of these types of assays for other cytotoxin-based clostridial antigens. This consultation took place on 9 and 10 March 2021.[7]

Laboratories involved in testing these products are strongly encouraged to use this study as a basis for developing and implementing their own tests.

The above recommendations, if successfully pursued, offer opportunities to significantly reduce animal usage, to shorten the duration of QC test procedures and to increase the precision of toxicity and antigenicity assays. This, in turn, will provide more accurate and consistent dosing of antigens in the final blended vaccines, help to promote compendial acceptance and to proffer a basis for improved international harmonisation across this area of product testing.

9. PARTICIPANTS (IN ALPHABETICAL ORDER BY COUNTRY)

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7 Weblink: https://www.edqm.eu/en/news/phasing-out-animal-testing-process-control-veterinary-vaccines-clostridium-septicum-proof.

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The BSP130 study was co-ordinated by Dr Marie-Emmanuelle Behr-Gross. Statistical analysis was performed by Arnold Daas in Part 1 and by David Le Tallec in Part 2. Sally Woodward provided organisational support as well as editorial and secretarial assistance in the preparation of the protocols and the reports. Louise Birrell and David Crowe are acknowledged for their support in the linguistic editing and layout work on this manuscript.

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11. ABBREVIATIONS

AM: arithmetic mean; BSP: Biological Standardisation Programme; CV: Coefficient of Variation; EC: European Commission; EDQM: European Directorate for the Quality of Medicines & HealthCare; EPAA: European Partnership for Alternative Approaches to Animal Testing; EU: European Union; GCV: Geometric Coefficient of Variation; GM: Geometric Mean; IS: International Standard; IU: International Unit; L+: Test dose determined using the lethal effect of a toxin in a toxin-antitoxin assay; LD: Lethal Dose; MLD: Minimum Lethal Dose; NBS: Nutrient Broth Saline; OD: Optical Density; OMCL: Official Medicines Control Laboratory; Ph. Eur.: European Pharmacopoeia; QC: Quality Control; SOP: Standard Operating Procedure; TCP: Total Combining Power; TNE+: Toxin Neutralisation Equivalence Plus; VCA: Vero Cell Assay.

12. LIST OF ANNEXES

Annex 1: Terminology and definitions

Annex 2: Table of recommended dilution ranges for C. septicum toxins and toxoids
Annex 3: In vitro MLD method, SOP
Annex 4: In vitro TNE+ method, SOP
Annex 5: In vitro TCP method, SOP
Annex 6: Statistical methods
Annex 7: Individual end-points from VC sensitivity assays
Annex 8: Individual end-points from latent toxicity assays
Annexes 9A and B: Intermediate end-points from TNE+ assays of the detector toxin (CSTx2) at 1.3-fold dilutions
Annexes 9C to E: Intermediate end-points from TNE+ assays of the detector toxin (CSTx2) at 1.1-fold dilutions
Annex 10: Final end-points from TNE+ evaluation of the test toxins in the 1.3-fold dilution assays
Annex 11: Final end-points (TCP Units) from the TCP evaluation of the test toxoids in the 10-increment dilution assays

13. ELECTRONIC ANNEXES

Electronic datasheets in Excel format were provided to the BSP130 Part 2 study participants for the following purposes:

- In vitro MLD determination of VC line sensitivity
- In vitro MLD determination of latent toxicity assessment of reference antiserum and test toxoids
- In vitro TNE+ determination of detector toxin toxicity
- In vitro TNE+ determination of test toxins toxicity
- In vitro TCP determination of the antigenicity of test toxoids

These reporting sheets are available upon request from the EDQM.

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ANNEX 1: TERMINOLOGY AND DEFINITIONS

GENERAL

L+ dose – the smallest quantity of a toxin that, in the conditions of the test, when mixed with 1 IU of antitoxin and administered by the specified route, causes the death of the test animals within a given period.

$L+_{50}$ dose – the smallest quantity of a toxin that, in the conditions of the test, when mixed with 1 IU of antitoxin and administered by the specified route, causes the death of 50 % of the test animals within a given period

LD_{50} – The statistically determined quantity of a substance that, when administered by the specified route, may be expected to cause the death of 50 per cent of the test animals within a given period.

Limit of quantitation – The lowest amount of the biologically active compound in a sample which can be quantitatively determined with appropriate precision and accuracy.

Precision – The closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions:

- Repeatability (inter-assay precision) expresses the precision under the same operating conditions over a short interval of time;
- Reproducibility (inter-laboratory precision) expresses the precision between results of several laboratories (collaborative studies).

Reference – An in-house preparation, the activity of which may be expressed relative to a standard preparation or in appropriate units derived from the test method.

Standard – A preparation of defined activity and composition available to any manufacturer, normally through a national or international authority.

Validation – The process by which the reliability and relevance of a procedure are established for a specific purpose.

STUDY SPECIFIC

Cell line end-point titre – The greatest dilution of toxin, or of a mixture containing toxin, that causes the death of more than 50 % of the cells.

Detecting toxin – *C. septicum* toxin supplied for use as the challenge or detector toxin in the TCP assays (Coded CSTx2).

Flat-bottomed tissue culture plate – Microtitre plate with flat-bottomed wells that is suitable for the culture of Vero cells.

Laboratory – The facility at which the assays are performed (coded 1 to 14).

Negative control – Microtitre plate wells containing Vero cells which have not been treated with *C. septicum* toxin.

Positive control – Microtitre plate wells containing Vero cells which have been treated with the detecting *C. septicum* toxin.

Residual toxicity tests on Vero cells – The determination of latent toxicity of toxoids/antisera, estimated by valid end-points (e.g. expressed as average number of dead wells in a row).

Sensitivity of Vero cells – The MLD of the detecting toxin (CSTx2).

Standard antitoxin – 3rd International Standard for *C. septicum* antitoxin, 500 IU per ampoule (VI). Derived from equine sera and established in 1957. For use in TCP assays.

Test toxin – *C. septicum* toxin samples supplied for assessment in the study (coded TxR, TxS, TxV, TxW, TxY and TxZ).

Test toxoid – *C. septicum* toxoid samples supplied for assessment in the study (coded TdA, TdC, TdD, TdN, TdO and TdP).

ANNEX 2: TABLE OF RECOMMENDED DILUTION RANGES FOR *C. SEPTICUM* TOXINS AND TOXOIDS

<table>
<tr><th>Sample code</th><th>Type of sample</th><th>Initial TNE+ dilution range</th><th>Initial TCP dilution range</th></tr>
<tr><td>CSTx2</td><td>Detector Toxin</td><td>525–5570
1.3-fold serial</td><td>N/A</td></tr>
<tr><td>TxR</td><td>Toxin</td><td rowspan="6">10–5120
2-fold serial</td><td rowspan="6">N/A</td></tr>
<tr><td>TxS</td><td>Toxin</td></tr>
<tr><td>TxV</td><td>Toxin</td></tr>
<tr><td>TxW</td><td>Toxin</td></tr>
<tr><td>TxY</td><td>Toxin</td></tr>
<tr><td>TxZ</td><td>Toxin</td></tr>
<tr><td>TdA</td><td>Toxoid</td><td rowspan="6">N/A</td><td>40-200/20 unit steps</td></tr>
<tr><td>TdC*</td><td>Toxoid</td><td>10-90/10 unit steps*</td></tr>
<tr><td>TdD</td><td>Toxoid</td><td>20-180/20 unit steps</td></tr>
<tr><td>TdN</td><td>Toxoid</td><td>20-180/20 unit steps</td></tr>
<tr><td>TdO</td><td>Toxoid</td><td>100-260/20 unit steps</td></tr>
<tr><td>TdP</td><td>Toxoid</td><td>140-300/20 unit steps</td></tr>
</table>

* The expected TCP value is very low, the first assay with 20 unit step dilutions can be omitted.
N/A: Not applicable
Note: The above dilution ranges are approximate, having been obtained from one laboratory on one occasion. They are not necessarily the most appropriate dilution ranges for all of these materials in every participant's testing system.

ANNEX 3: IN VITRO MLD METHOD

CLOSTRIDIUM SEPTICUM MINIMUM LETHAL DOSE (MLD) DETERMINATION BY CELL LINE ASSAY

OBJECTIVES

In vitro MLD assay is used for the following purposes:

1. To determine the sensitivity of VC lines to the toxic effects of *C. septicum* alpha toxin using the detector toxin, CSTx2;
2. To detect and quantify any residual *C. septicum* toxicity associated with the test toxoids and standard antitoxin (VI).

PRINCIPLES

The assay is intended to determine the toxicity of *C. septicum* toxin-containing materials on the basis of their lethality for Vero cells. With slight modifications, including the use of a *C. septicum* toxin of known toxicity, this assay can also be used to assess the sensitivity of different VC lines to *C. septicum* toxin.

In both of these assay types, dilutions of materials which may or are known to contain active *C. septicum* toxin, are applied to tissue culture plates containing confluent monolayers of Vero cells. Toxin present in the less diluted samples will kill the cells, whereas the more diluted samples, containing low levels or no toxin, will not kill the cells. The effect of the toxin on the cells can be visualised by staining the cells using Gram's crystal violet. The dead cells wash off

whereas the live cells adhere and are stained with the dye, which allows visual observation of the results and determination of the end-point titres.

The optical density (OD) of the wells is read. By comparing the ODs of the test sample wells with those of the negative control wells, end-point titres can be determined for the test samples. The end-point is expressed as a dilution: the greatest dilution of toxin that still causes death of more than 50 % of the cells.

HEALTH AND SAFETY

All manipulations involving direct contact between the cells and the environment must be done under aseptic conditions and performed within a Class 2 Safety cabinet. All antibiotics, anti-mycotics, test toxoids and the detector toxin should be treated as hazardous materials. Refer to manufacturer's safety data sheets for all reagents and materials used. All work should take place in a clean laboratory environment and laboratory coats should be worn.

EQUIPMENT

- Inverted microscope
- 96-well sterile 1 or 2 mL deep well plates with sterile lid (e.g. Eppendorf; Cat # 951032727 or #951033561 with #0030131525)
- Flat bottom tissue culture plates
- Sterile tubes (2, 15, 50 mL)
- 10, 25, 50 mL disposable plastic pipettes
- 10 µL, 200 µL and 1 mL single-channel pipette (Gilson or equivalent)
- 10 µL, 200 µL and 1 mL sterile pipette tips
- Clean plastic waste beaker (500 mL or equivalent)
- Multi-channel pipette (200 µL)
- Sterile reagent reservoirs
- Microtitre plate reader with appropriate software

MATERIALS

- Vero cells from the lowest passage number available
- Test toxoid samples
- *C. septicum* antitoxin, 3rd International Standard, (coded name VI) NIBSC
- Sterile detector *C. septicum* toxin, CSTx2
- Nutrient Broth Saline (NBS) 8.5 g NaCl in 200 mL nutrient broth (Oxoid/Thermo; Cat # CM0001 or equivalent) made up to 1 L with distilled water
- Maintenance medium (e.g. MEM Hanks)
- Culture medium (e.g. MEM Hanks) containing up to 10 % foetal calf serum (FCS)
- Gram's crystal violet solution
- Isopropyl alcohol (IPA)

PROCEDURE

General description of the experiment

The experiment is carried out on three consecutive days on two types of plate:

- deep-well dilution plate; and
- tissue culture plate.

Day 0. Vero cells are plated into 96-well culture plates at the appropriate density and put into a cell culture incubator for overnight incubation so that they will form a confluent layer on Day 1.

Day 1. First the growth medium is replaced on the culture plate with serum-free medium then reaction mixture is prepared on a deep-well dilution plate. Cells are treated with the described volume of the reaction mixture and the culture plates are then incubated overnight in a cell culture incubator.

Day 2. Evaluation of the results.

Preparation of culture plate (Day 0)

Vero cells in a culture flask are trypsinized and the cell suspension centrifuged. The cell pellet is then resuspended in culture medium containing up to 10 % FCS and the cell count is adjusted to 250 000 cell/mL. 100 µL of this suspension is pipetted into each well of the flat bottom tissue culture plate in the following setup:

Table 1 – *Layout of VC plating*

<table>
<tr><th></th><th>1</th><th>2</th><th>3</th><th>4</th><th>5</th><th>6</th><th>7</th><th>8</th><th>9</th><th>10</th><th>11</th><th>12</th></tr>
<tr><td>A</td><td colspan="12">UT (no cells, 100 µL medium/well)</td></tr>
<tr><td>B</td><td colspan="12" rowspan="7">25 000 cell/well in 100 µL culture medium (5 % FCS)</td></tr>
<tr><td>C</td></tr>
<tr><td>D</td></tr>
<tr><td>E</td></tr>
<tr><td>F</td></tr>
<tr><td>G</td></tr>
<tr><td>H</td></tr>
</table>

Note: Row A will be untreated blanks and contain no cells. This row contains medium only.

Incubate the plates in a humidified CO_2 incubator (37 °C, 5 % CO_2) for at least 20h.

If a study participant observes less than 90 % confluency on Day 1, adjust cell number in order to get the appropriate confluency. Prepare one culture plate for the sensitivity test and one culture plate for the residual toxicity determination of the six toxoids and the standard antitoxin.

Preparation of culture plate and dilution of test materials (Day 1)

a. Preparation of the culture plate

- Pre-warm maintenance medium and NBS at 37 °C in a water bath or incubator.
- Check the Vero cells in the tissue culture plates to ensure that they have formed confluent monolayers.
- Remove the cell growth medium from the cells by sharply flicking the plate contents into a suitable glass or plastic beaker, and aseptically replace with 100 µL of serum-free maintenance medium per well for all wells (including row A) using a multi-channel pipette.
- Return the plate to the CO2 incubator at 37 °C for 30 minutes to 2 hours.

b. Preparation of test samples

b/1. Determination of VC sensitivity using CSTx2

- Prepare a 1:8000 dilution of CSTx2 using sterile NBS in sterile tubes in 2 steps (e.g. 1st: 80x; 2nd: 100x).

- Place 200 µL of sterile NBS into all wells of columns 1-2 and 4-12 of a 1 or 2 mL sterile deep-well plate.
- Place 400 µL of the 1:8000 diluted CSTx2 into wells B3-C3-D3-E3-F3-G3.
- Prepare 2-fold serial dilutions of the test material using a multichannel pipette by transferring 200 µL from column 3, B-G → column 4, B-G. Mix by pipetting 5 times and continue the dilution from column 4 → column 5, etc. to column 12.

The plate setup is as follows:

Table 2 – *Dilution plate setup for Vero cell sensitivity assay*

	1	2	3	4	5	6	7	8	9	10	11	12
A	UT											
B	Neg	Neg	CSTx2 8000	16000	32000	64000	128000	256000	512000	1024000	2048000	4096000
C	Neg	Neg	CSTx2 8000	16000	32000	64000	128000	256000	512000	1024000	2048000	4096000
D	Neg	Neg	CSTx2 8000	16000	32000	64000	128000	256000	512000	1024000	2048000	4096000
E	Neg	Neg	CSTx2 8000	16000	32000	64000	128000	256000	512000	1024000	2048000	4096000
F	Neg	Neg	CSTx2 8000	16000	32000	64000	128000	256000	512000	1024000	2048000	4096000
G	Neg	Neg	CSTx2 8000	16000	32000	64000	128000	256000	512000	1024000	2048000	4096000
H	Neg	Neg	NBS									

Note: The numbers represent the fold dilution in a given well.

b/2. Determination of residual toxicity of test toxoids and reference antitoxin

- Prepare 1:5 dilutions of test toxoids in sterile NBS.
- Prepare 5 IU/mL solution of the standard antitoxin in sterile NBS.
- Place 200 µL of sterile NBS into all wells of column 1-2 and 4-12 of a 1 or 2 mL sterile deep-well plate.
- Place 400 µL of the 1:5 diluted toxoids and the 5 IU/mL antitoxin into wells B3-H3.
- Prepare two-fold serial dilutions of the test material using a multichannel pipet by transferring 200 µL from column 3, B-H → column 4, B-H. Mix by pipetting 5 times and continue the dilution from column 4 → column 5, etc. to column 12.

The plate setup is as follows:

Table 3 – *Dilution plate setup of residual toxicity assay*

	1	2	3	4	5	6	7	8	9	10	11	12
A	UT											
B	Neg	Neg	TdA, 5	10	20	40	80	160	320	640	1280	2560
C	Neg	Neg	TdC, 5	10	20	40	80	160	320	640	1280	2560
D	Neg	Neg	TdD, 5	10	20	40	80	160	320	640	1280	2560
E	Neg	Neg	TdN, 5	10	20	40	80	160	320	640	1280	2560
F	Neg	Neg	TdO, 5	10	20	40	80	160	320	640	1280	2560
G	Neg	Neg	TdP, 5	10	20	40	80	160	320	640	1280	2560
H	Neg	Neg	antitoxin, 5	2.5	1.25	0.625	0.3125	0.1563	0.0781	0.0391	0.0195	0.0098

Note: The numbers represent the fold dilution and IU/mL concentration for toxoids and antitoxin, respectively, in a given well.

c. Inoculation of Vero cells with test material dilutions

- Remove the culture plate from the incubator.
- Inoculate the culture plate with 100 µL samples from the appropriate dilution plate using a multi-channel pipette. Start with column 1-2, and then column 12 then work backwards across the plate until column 3, hence, replicating exactly the layout of the dilution plate.
- Incubate the plates in a CO2 incubator at 37 °C for 18-24 hours.

Evaluation of the experiment (Day 2)

Microscopic observation

- Check the cells in the negative control wells under inverted microscope.
- Stain the Vero cells on the plates as follows:

Cell staining

- Remove the medium from the wells by sharply flicking the plate contents into a suitable glass or plastic beaker.
- Add 50 µL crystal violet solution per well using a multi-channel pipette. Incubate for 5 minutes at room temperature then remove the stain from the wells by sharply flicking the plate contents into a suitable glass or plastic beaker.
- Gently rinse all wells at least four times with tap water by dipping the plate into water then flick the water off.
- Leave the plate without lid on for 30 min to dry.
- Add 100 µL of IPA per well using a multi-channel pipette. Cover the plate with a lid and incubate at room temperature for 30 minutes with occasional gentle agitation until crystal violet fully dissolves.

Data interpretation

a. OD reading and calculations (as performed by the Excel reporting sheet).

- Read the OD of the plate at 570 or 630 nm (450 nm reference).
- From the values measured at 570 or 630 nm subtract the OD values measured at 450 nm (reference wavelength) in the corresponding wells. Then the median OD of UT wells is subtracted from the corrected OD of each well.
- For each plate the median OD value for the negative controls is calculated.
- 50 % of the median value of the negative controls is calculated and this is the cut-off value.
- The end-points for the detector toxin and the toxoid test samples are determined as the well with the highest dilution of material which has an OD below the cut-off value.
- Example: When the median absorbance of the negative control wells was:

– Negative control median = 0.804
– 50 % of the median = 0.402
– Cut-off point = 0.402

All the wells with absorbance lower than the cut-off value (< 0.402) were affected by the test material.

- Record the end-point values for each replicate.
- The results are to be reported in the reporting sheet *BSP130 Vero Cell Sensitivity Confirmation*.

b. Validity criteria

- The coefficient of variation (CV) of the cell negative control is equal to or less than 20 %.

- The end-point values obtained in the Vero cell sensitivity assay, must not differ by more than one well.

ANNEX 4: *IN VITRO* TNE+ METHOD

CLOSTRIDIUM SEPTICUM TOXIN NEUTRALISATION EQUIVALENCE PLUS (TNE+) DETERMINED BY CELL LINE ASSAY

OBJECTIVES

In vitro TNE+ assay is used for the following purposes:

1. **To establish the toxin neutralisation equivalence plus (TNE+) of CSTx2**. A first assay is carried out using 1.3-fold dilution steps. Then, the resulting TNE+ value will be refined in a second series of assays using 1.1-fold dilution step in three independent experiments. The geometric mean (GM) of the 3 valid results will then be used as the CSTx2 TNE+ value in TNE+ assays for the test toxins and TCP assays later in the study.
2. **To determine the TNE+ value of the C. septicum test toxins**. Test toxins will be measured once in a 2-fold dilution step. The next step is to refine the TNE+ value by repeating the assay at 1.3-fold steps, starting at the penultimate dilution giving the last positive response in the 2-fold dilution step assay. The aim is to report the results of one valid assay performed using the 2-fold dilution range and three valid assays performed using the 1.3-fold dilution range. CSTx2 will be used as internal control in a 1.1-fold dilution using the participant lab's own TNE+ value. The dilution range of CSTx2 should include 4 steps before and 5 steps after the dilution corresponding to the predetermined TNE+ value.

PRINCIPLES

The assay is intended to determine the toxicity of *C. septicum* toxins on the basis of how much the toxin has to be diluted before loss of lethality on Vero cells when the toxin is pre-incubated with a set amount of antitoxin. In this assay, dilutions of the *C. septicum* toxins are mixed with a set amount of antitoxin and incubated. The mixtures are then applied to a tissue culture plate containing confluent monolayers of Vero cells.

In the mixtures containing the less diluted toxin samples, there will be insufficient antitoxin to neutralise the toxin that is present, whereas in the mixtures containing the more diluted toxin samples, the amount of antitoxin present will be sufficient to neutralise the toxin. The presence of unneutralised toxin can be detected by its lethal effect on the Vero cells. The effect of the mixtures on the cells can be visualised by staining the cells using Gram's crystal violet. The dead cells wash off whereas the live cells adhere and are stained with the dye, which allows visual observation of the results and determination of the end-points.

The optical density (OD) of the wells is read. By comparing the ODs of the test sample wells with those of the negative control wells, end-points can be determined for the test samples. The end-point is the mixture containing the greatest dilution of toxin that still causes death of more than 50 % of the cells. The toxicity of a test toxin can then be expressed in IU/mL relative to the standard antitoxin.

HEALTH AND SAFETY

All manipulations involving direct contact between the cells and the environment must be done under aseptic conditions and performed within a Class 2 Safety cabinet. All antibiotics, antimycotics, test toxins and the detector toxin should be treated as hazardous materials. Refer to manufacturer's safety data sheets for all reagents and materials used. All work should take place in a clean laboratory environment and laboratory coats should be worn.

EQUIPMENT

- Inverted microscope

- 96-well sterile 1 or 2 mL deep well plates with sterile lid (e.g. Eppendorf; Cat # 951032727 or #951033561 with #0030131525)
- Flat bottom tissue culture plates
- Sterile tubes (2, 15, 50 mL)
- 10, 25, 50 mL disposable plastic pipettes
- 10 μL, 200 μL and 1 mL single-channel pipette (Gilson or equivalent)
- 10 μL, 200 μL and 1 mL sterile pipette tips
- Clean plastic waste beaker (500 mL or equivalent)
- Multi-channel pipette (200 μL)
- Sterile reagent reservoirs
- Microtitre plate reader with appropriate software

MATERIALS

- Vero cells from the lowest passage number available
- Test toxin samples
- *C. septicum* antitoxin, 3rd International Standard, (coded name VI) NIBSC
- Sterile detector *C. septicum* toxin, CSTx2
- Nutrient Broth Saline (NBS) 8.5 g NaCl in 200 mL nutrient broth (Oxoid/Thermo; Cat # CM0001 or equivalent) made up to 1 L with distilled water
- Culture medium (e.g. MEM Hanks) containing up to 10 % foetal calf serum (FCS)
- Maintenance medium (e.g. MEM Hanks)
- Gram's crystal violet solution
- Isopropyl alcohol (IPA)

PROCEDURE

General description of the experiment

The experiment is carried out on three consecutive days on two types of plate:

i. deep-well dilution plate and
ii. two replicate tissue culture plates for each dilution plate

Day 0. Vero cells are plated into 96-well culture plates at the appropriate density and put into a cell culture incubator for overnight incubation so that they will form a confluent layer on Day 1.

Day 1. First, the growth medium is replaced on the culture plate with serum-free medium then reaction mixture is prepared on a deep-well dilution plate. Cells are treated with the described volume of reaction mixture and the culture plates are then incubated overnight in a cell culture incubator.

Day 2. Evaluation of the results.

Preparation of the culture plate (Day 0)

Vero cells in a culture flask are trypsinized and the cell suspension centrifuged. The cell pellet is then resuspended in culture medium containing up to 10 % FCS and the cell count is adjusted to 250 000 cell/mL. 100 μL of this suspension is pipetted into each well of the flat bottom tissue culture plate in the following setup:

Table 4 – *Layout of Vero cell plating*

	1	2	3	4	5	6	7	8	9	10	11	12
A	UT (no cells, 100 µL medium/well)											
B–H	25 000 cell/well in 100 µL culture medium (5 % FCS)											

Note: Row A will be untreated blanks and contain no cells. This row contains medium only.

Incubate the plates in a humidified CO_2 incubator (37 °C, 5 % CO_2) for at least 20h.

If less than 90 % confluency on Day 1 is observed, adjust cell number in order to get the appropriate confluency.

Prepare two parallel culture plates (replicates) for each dilution plate.

Preparation of culture plate and dilution of test materials (Day 1)

a. Preparation of the culture plate

- Pre-warm maintenance medium at 37 °C in a water bath or incubator.
- Check the Vero cells in the tissue culture plates to ensure that they have formed confluent monolayers.
- Remove the cell growth medium from the cells by sharply flicking the plate contents into a suitable glass or plastic beaker, and aseptically replace with 100 µL of serum-free maintenance medium per well for all wells (including row A) using a multi-channel pipette.
- Return the plate to the CO_2 incubator at 37 °C for 30 minutes to 2 hours. The next step is either b1 or b2 depending on which assay is being performed:

b1. Establishment of the CSTx2 TNE+ value – Preparation of 1.3-fold step and 1.1-fold step serial dilutions of the CSTx2 detector toxin

First assay:

Prepare a 10 step 1.3-fold serial dilution series of CSTx2 starting from a 1:525 pre-dilution using sterile NBS in 50 mL tubes:

- prepare 12 mL of 1:525 diluted CSTx2 (add 22.9 µL CSTx2 into 12 mL NBS) - mix by pipetting
- put 2.4 mL of NBS into each tube from 2 to 10
- from tube 1 transfer 8 mL solution into tube 2, mix by pipetting
- continue this serial dilution from tube 2 to tube 10

Second assay:

Prepare a 10 step 1.1-fold serial dilution series of CSTx2 using sterile NBS and 50 mL tubes starting at the penultimate dilution giving the last positive response in the 1.3-fold dilution step assay. The Excel reporting sheet generates the penultimate dilution on the “Instruction for use” sheet.

- prepare 30 mL of CSTx2 at the starting dilution - mix by pipetting
- put 2.5 mL NBS into each tube from 2 to 10
- from tube 1 transfer 25 mL solution into tube 2, mix by pipetting continue this serial dilution from tube 2 to tube 10

b2. Toxicity assay for the test toxins – Preparation of test toxin samples and the detector toxin

First assay:

Prepare serial dilutions of the test toxin samples using sterile NBS in sterile tubes (15 mL or 50 mL, depending on the volume of the dilution steps). These are ten step 2-fold serial dilutions starting from 1:10 pre-dilutions of the supplied test toxins.

Prepare a serial dilution of CSTx2, as control, using the previously established TNE+ value as the approximate central dilution in a range of ten 1.1-fold step dilutions.

Second assay:

Repeat the first assay but, this time, prepare 1.3-fold serial dilutions of the test toxins, starting with the penultimate dilutions giving the last positive response in the 2-fold dilution step assay.

Prepare a serial dilution of CSTx2, as control, using the previously established TNE+ value as the approximate central dilution in a range of ten 1.1-fold step dilutions.

c. Preparation of antitoxin dilution

Prepare a 0.2 IU/mL concentration of antitoxin from the previously aliquoted 50 IU/mL stock solution using sterile NBS in a sterile 50 mL tube. 18 mL of diluted antitoxin is sufficient for one dilution plate.

d. Loading the dilution plates

- Add 500 µL of sterile NBS to each of the wells in columns 1 and 2 and rows A and H of a deep-well plate (highlighted in light grey in Tables 6 and 7).
- Add 250 µL of each toxin dilution into the appropriate well across the dilution plate with the lowest dilution of the toxin in column 3 and the highest dilution in column 12.
- Add 250 µL of 0.2 IU/mL antitoxin dilution into each of the wells B3-G12.
- Gently tap the plate by hand to mix and incubate the mixtures at 37 °C for 1 h.

Table 5 – *Plate setup for CSTx2 TNE+ value establishment assay at 1.3-fold dilutions*

	1	2	3	4	5	6	7	8	9	10	11	12
A	UT											
B	Neg	Neg	CSTx2, 525	683	887	1153	1499	1949	2534	3294	4283	5567
C	Neg	Neg	CSTx2, 525	683	887	1153	1499	1949	2534	3294	4283	5567
D	Neg	Neg	CSTx2, 525	683	887	1153	1499	1949	2534	3294	4283	5567
E	Neg	Neg	CSTx2, 525	683	887	1153	1499	1949	2534	3294	4283	5567
F	Neg	Neg	CSTx2, 525	683	887	1153	1499	1949	2534	3294	4283	5567
G	Neg	Neg	CSTx2, 525	683	887	1153	1499	1949	2534	3294	4283	5567
H	Neg	Neg	NBS									

Neg: =negative control wells, UT=untreated wells. The numbers represent the fold dilutions in the wells.

Table 6 – *Plate setup for test toxin TNE+ determination at 2-fold dilutions*

	1	2	3	4	5	6	7	8	9	10	11	12
A	UT											
B	Neg	Neg	Tx1, 10	20	40	80	160	320	640	1280	2560	5120
C	Neg	Neg	Tx1, 10	20	40	80	160	320	640	1280	2560	5120
D	Neg	Neg	Tx2, 10	20	40	80	160	320	640	1280	2560	5120
E	Neg	Neg	Tx2, 10	20	40	80	160	320	640	1280	2560	5120
F	Neg	Neg	CSTx2, 1020	1122	1234	1358	1493	1643	1807	1988	2186	2405
G	Neg	Neg	CSTx2, 1020	1122	1234	1358	1493	1643	1807	1988	2186	2405
H	Neg	Neg	NBS									

Neg: = negative control wells, UT = untreated wells. The numbers represent the fold dilutions in the wells. In this assay, CSTx2 is always used at 1.1-fold dilution steps as control.

e. Inoculation of Vero cells with toxin/antitoxin mixtures

- Remove the culture plates from the incubator (two culture plates for each dilution plate).
- Inoculate the culture plates with 100 μL samples from the dilution plate using a multi-channel pipette. Start with column 1 and 2 followed by column 12 and then work backwards across the plate from column 12 to column 3, hence, replicating exactly the layout of the dilution plate.
- Incubate the plates in a CO_2 incubator at 37 °C for 18-24 hours.

Evaluation of the experiment (Day 2)

Microscopic observation

- Check the cells in the negative control wells under inverted microscope.
- Stain the Vero cells on the plates as follows:

Cell staining

- Remove the medium from the wells by sharply flicking the plate contents into a suitable glass or plastic beaker.
- Add 50 μL crystal violet solution per well using a multi-channel pipette. Incubate for 5 minutes at room temperature then remove the stain from the wells by sharply flicking the plate contents into a suitable glass or plastic beaker.
- Gently rinse all wells at least four times with tap water by dipping the plate into water then flick the water off.
- Leave the plate without lid on for 30 min to dry.
- Add 100 μL of IPA per well using a multi-channel pipette. Cover the plate with a lid and incubate at room temperature for 30 minutes with occasional gentle agitation until crystal violet fully dissolves.

Data interpretation

a. OD reading and calculations (as performed by the Excel reporting spread sheet).

- Read the OD of the plate at 570 or 630 nm (450 nm reference).
- From the values measured at 570 or 630 nm subtract the OD values measured at 450 nm (reference wavelength) in the corresponding wells. Then the median OD of UT wells is subtracted from the corrected OD of each well.
- For each plate, the median OD value for the negative controls is calculated.
- 50 % of the median value of the negative controls is calculated and this is the cut-off value.

- The end-points are determined as the wells with the highest dilutions of toxins which have ODs below the cut-off value.
- Example: When the median absorbance of the negative control wells was:

– Negative control median = 0.804

– 50 % of the median = 0.402

– Cut-off point = 0.402

- All the wells with absorbance lower than the cut-off value (< 0.402) were affected by the toxin.
- Record the cut-off values for each replicate.
- The results are to be reported in the reporting sheets: *BSP130 TNE Value of Detector Toxin.xlsx* and *BSP130 TNE Value of Toxins*, as appropriate.

b. Validity Criteria

For CSTx2

- The coefficient of variation (CV) of the cell negative control is equal to or less than 20 %.
- The end-points of the 6 replicate rows within plate must not differ by more than two wells.
- The mean end-point values of the replicate plates must not differ by more than two dilution steps (= dilution factor to the 2nd; in the case of 1.1 × dilution this translates into 1.12 = 1.21).

For test toxins

- The coefficient of variation (CV) of the cell negative control is equal to or less than 20 %.
- The end-point for the detector toxin must be the same as in the TNE+ determination assay or not more than two dilution steps either side.
- The end-points of the 2 replicate rows within the plate must not differ by more than two wells.
- The mean end-point values of the replicate plates must not differ by more than two dilution steps (= dilution factor to the 2nd; in the case of 1.3× dilution this translates into 1.32 = 1.69).

c. TNE+ calculation from the end-point dilution values:

The TNE+ end-point for the toxin is the well containing the greatest dilution of toxin that, when mixed with the set amount of standard antitoxin, still shows greater than 50 % cell death.

As each 500 µL reaction mixture contains 250 µL toxin and 250 µL of antitoxin, the final dilution factor of the toxin in the reaction mixture is the dilution (applied when preparing each dilution step) multiplied by 2.

The set concentration of the antitoxin that is to neutralise the test toxin in each reaction mixture is 0.1 IU/mL. The TNE+ value of the toxin is determined in terms of IU/mL antitoxin, therefore the final dilution at the end-point (2 × the end-point dilution value) of the toxin has to be presented in terms of IU/mL antitoxin. Thus, the TNE+ value is calculated by dividing the reciprocal of the final dilution value at the end-point by 10 (i.e. in practical terms the TNE+ value is the reciprocal of the end-point dilution value multiplied by 0.2).

In the following example, based on the layout in Table 7, the cut-off well for toxin 1 is in column 10. Therefore, the TNE+ value of the undiluted toxin 1 is:

The reciprocal of the end-point dilution × 2 (to give the final dilution) × 0.1 (to give IU/mL)

$$= 128 \times 2 \times 0.1 = 128 \times 0.2 = 25.6 \text{ IU/mL.}$$

ANNEX 5: *IN VITRO* TCP METHOD

CLOSTRIDIUM SEPTICUM TOTAL COMBINING POWER DETERMINATION BY CELL LINE ASSAY

OBJECTIVE

In vitro TCP is used to measure the antigenicity of *C. septicum* test toxoids using a Vero cell line assay.

PRINCIPLES INVOLVED

The Total Combining Power (TCP) assay is used to measure the antigenicity of *C. septicum* toxoid. Dilutions of toxoid samples are mixed and incubated with a known concentration of neutralising antitoxin and then with dilutions of a detector toxin. In the cell line-based TCP assay the mixtures are applied to a tissue culture plate containing confluent monolayers of Vero cells. At low dilutions the toxoid should be able to bind all neutralising antibodies leaving detector toxin free in solution which in turn causes cell death. The cytotoxic effect decreases with increasing toxoid dilutions until a point is reached (i.e. the end-point of the assay) when the cells no longer die as the whole amount of detector toxin is bound by the neutralising antibodies.

The effect of the mixture on the cells can be visualised by direct observation under the microscope as well as by staining the cells using Gram's crystal violet. The dead cells wash off the plate surface, whereas the live cells remain adhered and are stained with the dye, which allows determination of the results by reading the optical density (OD) of the wells. By comparing the ODs of the test sample wells with those of the negative control wells, end-points and thus TCP values of the test samples can be determined.

The cross-titration of both the test toxoid and the detector toxin on the plate enables the determination of the TCP value of the test toxoid independently of the sensitivity of the cells to the toxin, exactly at the point where the detector toxin is in equilibrium with the antitoxin (i.e. at the TNE+ value of the detector toxin).

This assay can be used to determine the antigenicity of *C. septicum* toxoid-containing materials.

The test is performed in two consecutive steps:

- initial assay with 20-increment dilution steps
  The initial dilution for each test toxoid is given in Table 1.
- refinement assay with 10-increment dilution steps
  The initial toxoid dilution in this second step is determined by the end-point dilution obtained in the initial assay. That end-point dilution is placed at the centre of a 9-well dilution range on the plate so that there are four dilutions before and four dilutions after that end-point dilution. The first dilution of this range is most easily calculated by subtracting a value of 40 increments from the initial assay's end-point dilution.

HEALTH AND SAFETY

All manipulations involving direct contact between the cells and the environment must be done under aseptic conditions and performed within a Class 2 Safety cabinet. All antibiotics, antimycotics and the CSTx2 detector toxin should be treated as hazardous materials. Refer to manufacturer's safety data sheets for all reagents and materials used. All work should take place in a clean laboratory environment and laboratory coats should be worn.

EQUIPMENT

- Inverted microscope
- 96-well sterile 1 or 2 mL deep well plates with sterile lid (e.g. Eppendorf; Cat # 951032727 or #951033561 with #0030131525)
- Flat bottom tissue culture plates

- Sterile tubes (2, 15, 50 mL)
- 10, 25, 50 mL disposable plastic pipettes
- 10 µL, 200 µL and 1 mL single-channel pipette (Gilson or equivalent)
- 10 µL, 200 µL and 1 mL sterile pipette tips
- Clean plastic waste beaker (500 mL or equivalent)
- Multi-channel pipette (200 µL)
- Sterile reagent reservoirs
- Microtitre plate reader with appropriate software

MATERIALS

- Vero cells from the lowest passage number available
- Test toxoid samples
- *C. septicum* antitoxin, 3rd International Standard, (coded name VI) NIBSC
- Sterile detector *C. septicum* toxin, CSTx2
- Nutrient Broth Saline (NBS) 8.5 g NaCl in 200 mL nutrient broth (Oxoid/Thermo; Cat # CM0001 or equivalent) filled up to 1 L with distilled water
- Culture medium (e.g. MEM Hanks) containing up to 10 % foetal calf serum (FCS)
- Maintenance medium (e.g. MEM Hanks)
- Gram's crystal violet solution
- Isopropyl alcohol (IPA)

PROCEDURE

General description of the experiment

The experiment is carried out on three consecutive days on two types of plate:

i. deep-well dilution plate and

ii. two replicate tissue culture plates

Day 0. Vero cells are plated into 96-well culture plates at the appropriate density and put into a cell culture incubator for overnight incubation so that they will form a confluent layer on Day 1.

Day 1. First, the growth medium is replaced on the culture plate with serum-free medium then reaction mixture is prepared on a deep-well dilution plate. Cells are treated with the described volume of the reaction mixture and the cell plates are then incubated overnight in a cell culture incubator.

Day 2. Evaluation of the results.

Preparation of culture plate (Day 0)

Vero cells in a culture flask are trypsinised and the cell suspension centrifuged. The cell pellet is then resuspended in culture medium containing up to 10 % FCS and the cell count is adjusted to 250 000 cell/mL. 100 µL of this suspension is pipetted into each well of the flat bottom tissue culture plate in the following setup:

Table 7 – *Layout of Vero cell plating*

	1	2	3	4	5	6	7	8	9	10	11	12
A	UT (no cells, 100 µL medium/well)											
B	25 000 cell/well in 100 µL culture medium (5 % FCS)											
C												
D												
E												
F												
G												
H												

Note: Row A will be untreated blanks and contain no cells. This row contains medium only.

The plates are then put into a humidified CO_2 incubator (37 °C, 5 % CO_2) for at least 20 h.

If a study participant observes less than 90 % confluency on Day 1, the cell number should be adjusted accordingly to get the appropriate confluency. For one dilution plate two culture plates (replicates) are prepared.

Preparation of culture plate and dilution of test materials (Day 1)

a. Preparation of the reaction mixture in the dilution plate

A 96-well, deep-well microplate containing the reaction mixture is prepared. The reaction is made up of two consecutive reaction steps. First, the standard antitoxin is incubated with different concentrations of the toxoid sample for 1 hour and then the detector toxin is added at different dilutions and the 3-component mixture is incubated for a further 1 hour. The toxoid sample is diluted horizontally across the plate, whereas the detector toxin is diluted vertically down the plate.

For each toxoid sample, a separate dilution plate is needed. As three dilution plates (i.e. three toxoids) can be conveniently handled in one experimental session, the volumes given as examples for the antitoxin and detector toxin in the sections below are always calculated for three dilution plates.

b. Preparation and addition of standard antitoxin

- Prepare two solutions of the rehydrated *C. septicum* International Standard antitoxin using sterile NBS. One solution at a final concentration of 0.4 IU/mL and the other at a final concentration 0.8 IU/mL.
- Add 125 µL of *C. septicum* antitoxin diluted to 0.4 IU/mL to wells B-H in column 3 (for three dilution plates, i.e. three toxoids, a total volume of 4 mL is usually sufficient for dispensing from a reservoir for this step).
- Add 125 µL of *C. septicum* antitoxin diluted to 0.8 IU/mL to wells B-H in column 4 to column 12 (for three dilution plates, i.e. three toxoids, a total volume of 33 mL is usually sufficient for dispensing from a reservoir for this step).
- Add 125 µL NBS to wells B-H in columns 1 and 2.
- Add 125 µL NBS to all the wells in row A.

See table below for plate layout:

Table 8 – *Layout for the addition of antitoxin and NBS to the dilution plate*

<table>
<tr><td>Antitoxin</td><td>1</td><td>2</td><td>3</td><td>4</td><td>5</td><td>6</td><td>7</td><td>8</td><td>9</td><td>10</td><td>11</td><td>12</td></tr>
<tr><td>A</td><td colspan="12">NBS</td></tr>
<tr><td>B</td><td colspan="2" rowspan="7">NBS</td><td rowspan="7">0.4 IU/mL anti-toxin</td><td colspan="9" rowspan="7">0.8 IU/mL antitoxin</td></tr>
<tr><td>C</td></tr>
<tr><td>D</td></tr>
<tr><td>E</td></tr>
<tr><td>F</td></tr>
<tr><td>G</td></tr>
<tr><td>H</td></tr>
</table>

c. Preparation and addition of test toxoid

The TCP is determined from a dilution range, where the toxoid is diluted in a linear fashion. This means that the increments between dilution points are the same all across the range (e.g. × 10, × 20, × 30, × 40 dilutions). The initial dilution for each test toxoid should be based on the appropriate dilution range recommended in Table 1.

- Prepare 9 different individual dilutions of the test toxoid.
- Add 125 µL of the individual dilutions to wells B-H from columns 4 to 12, starting with the highest dilution in column 12 and ending with the lowest dilution in column 4 (a net volume of 875 µL is needed for each toxoid dilution, so a total volume of 2 mL is usually sufficient for dispensing from a reservoir; if pipetting from the highest dilution to the lowest the same pipette tips can be used for dispensing).
- Add 125 µL NBS to the wells B-H in columns 1, 2 and 3.
- Add 125 µL NBS to all of the wells in row A.
- Gently tap the plate by hand to mix and incubate the mixtures at 37 °C for 1 h.

See table below for plate layout.

Table 9 – *Layout for the addition of the test toxoid dilutions and NBS on the dilution plate*

<table>
<tr><td>Toxoid</td><td>1</td><td>2</td><td>3</td><td>4</td><td>5</td><td>6</td><td>7</td><td>8</td><td>9</td><td>10</td><td>11</td><td>12</td></tr>
<tr><td>A</td><td colspan="12">NBS</td></tr>
<tr><td>B</td><td colspan="3" rowspan="7">NBS</td><td rowspan="7">Dil1</td><td rowspan="7">Dil2</td><td rowspan="7">Dil3</td><td rowspan="7">Dil4</td><td rowspan="7">Dil5</td><td rowspan="7">Dil6</td><td rowspan="7">Dil7</td><td rowspan="7">Dil8</td><td rowspan="7">Dil9</td></tr>
<tr><td>C</td></tr>
<tr><td>D</td></tr>
<tr><td>E</td></tr>
<tr><td>F</td></tr>
<tr><td>G</td></tr>
<tr><td>H</td></tr>
</table>

d. Preparation and addition of detector toxin (CSTx2)

The detector toxin is serially diluted with a dilution factor of 1.1. The dilution range should include three steps before and three steps after the dilution corresponding to the TNE+ value of the detector toxin (CSTx2).

- Prepare seven different dilutions of the detector toxin.

  To simplify the practical work and spare reagent start with calculating the volume needed for the highest dilution and then continue with the volume calculation of the previous points taking into account the extra volume needed for transferring from the previous point.

  Example for a dilution range with a starting dilution of 1250x:

Tube 1: Dilute 40 µL of detector toxin in 44.96 mL NBS in multiple steps in Tube 1.

Tube 2: Transfer 41 mL from Tube 1 to 4.1 mL NBS in Tube 2, mix thoroughly.

Tube 3: Transfer 36 mL from Tube 2 to 3.6 mL NBS in Tube 3, mix thoroughly.

Tube 4: Transfer 30 mL from Tube 3 to 3 mL NBS in Tube 4, mix thoroughly.

Tube 5: Transfer 25 mL from Tube 4 to 2.5 mL NBS in Tube 5, mix thoroughly.

Tube 6: Transfer 17 mL from Tube 5 to 1.7 mL NBS in Tube 6, mix thoroughly.

Tube 7: Transfer 9 mL from Tube 6 to 0.9 mL NBS in Tube 7, mix thoroughly.

- Add 250 µL of the individual dilutions to the columns 3-12 from rows B to H, starting with the highest dilution in row H and ending with the lowest dilution to row B.
- A net volume of 7.5 mL is needed for each dilution for three dilution plates, i.e. for three test toxoids, so a total volume of 9 mL is usually sufficient for conveniently dispensing from a reservoir. It is crucial to change tips between adjacent rows in order to avoid cross-contamination.
- Add 250 µL NBS to the wells B-H in columns 1 and 2.
- Add 250 µL NBS to the entire row A.
- Gently tap the plate by hand to mix and incubate the mixtures at 37 °C for 1 h.

See table below for plate layout.

Table 10 – *Layout for the detector toxin dilutions on the dilution plate*

Detector toxin	1	2	3	4	5	6	7	8	9	10	11	12
A	NBS											
B	NBS		Dilution #1									
C			Dilution #2									
D			Dilution #3									
E			Dilution #4									
F			Dilution #5									
G			Dilution #6									
H			Dilution #7									

e. Preparation of the culture plate.

Immediately after detector toxin is added to the dilution plate and the 2nd incubation starts, the culture medium on the culture plate is changed to maintenance medium (without FCS).

- Pre-warm maintenance medium and NBS at 37 °C in a water bath or incubator.
- Check the cells in the microtitre plates to ensure that they have formed confluent monolayers.
- Remove the cell growth medium from the cells by sharply flicking the plate contents into a suitable glass or plastic beaker, and aseptically replace it with 100 µL of maintenance medium per well using a multi-channel pipette.
- Return the plate to the CO_2 incubator at 37 °C for 30 to 60 minutes.

f. Inoculation of Vero cells with the reaction mixtures

- Remove the culture plates from the incubator (two replicate plates per toxoid).
- Inoculate the culture plates with 100 µL samples from the dilution plate using a multi-channel pipette. Start with row A followed by row H and then work up the plate from row H to row B, hence replicating exactly the layout of the dilution plate.
- Incubate the plates in a CO_2 incubator at 37 °C for 18–24 hours.

Evaluation of the experiment (Day 2)

Microscopic observation

- Check the cells in the negative control wells under an inverted microscope.
- Stain the Vero cells on the plates as follows:

Cell staining

- Remove the medium from the wells by sharply flicking the plate contents into a suitable glass or plastic beaker.
- Add 50 µL crystal violet solution per well using a multi-channel pipette. Incubate for 5 minutes at room temperature then remove the stain from the wells by sharply flicking the plate contents into a suitable glass or plastic beaker.
- Gently rinse all wells at least four times with tap water by dipping the plate into water then flick the water off.
- Leave the plate without lid on for 30 min to dry.
- Add 100 µL of IPA per well using a multi-channel pipette. Cover the plate with a lid and incubate at room temperature for 30 minutes with occasional gentle agitation until crystal violet fully dissolves.

Data interpretation

a. OD reading and calculations (as performed by the Excel reporting spread sheet)

- Read the OD of the plate at 570 or 630 nm (450 nm reference).
- From the values measured at 570 or 630 nm subtract the OD values measured at 450 nm (reference wavelength) in the corresponding wells. Then the median OD of UT wells is subtracted from the corrected OD of each well.
- For each plate the median OD value for the negative controls is calculated.
- 50 % of the median value of the negative controls is calculated and this is the cut-off value.
- The end-point for the detector toxin in Column 3 is determined as the well with the highest dilution of toxin which has an OD below the cut-off value.
- The end-point for the toxoid test sample is in the same row where the end-point for the detector toxin was found and is determined as the highest dilution of toxoid which has an OD value below the cut-off value.
- Example: When the median absorbance of the negative control wells was:

– Negative control median = 0.804

– 50 % of the median = 0.402

– Cut-off point = 0.402

All the wells with absorbance lower than the cut-off value (< 0.402) were affected by the detector toxin.

- Record the cut-off values for each replicate plate.
- The results are to be reported in the reporting sheet “BSP130 Antigenicity of Toxoids”.

b. Validity criteria

- The coefficient of variation (CV) of the cell negative control is equal to or less than 20 %.
- The end-point values for the detector toxin CSTx2 must be the same as determined in step III or must not differ by more than two dilution steps either side (vertically).
- The end-point values of the replicate plates must not differ by more than two wells.

c. Identification of appropriate toxoid end-point dilution value and TCP calculation

Each of the wells in column 3 of the dilution plate contain the antitoxin at a final concentration of 0.1 IU/mL. Moving down column 3 each well contains the detector toxin (CSTx2) at an increasing dilution. Column 3 is, in effect, acting as a check on the TNE+ value for the detector toxin in each culture plate (see In vitro TNE+ method, Annex 4). Therefore, the end-point in column 3 is the well which most closely predicts the TNE+ value for the detector toxin in this system. As the same concentration of detector toxin is present in the wells across this row it is the end-point on this row which will most accurately quantify the TCP value for the toxoid. For example, if the end-point in column 3 is well 3F and the end-point across the toxoid dilutions in row F is well 7F, the concentration of toxoid in well 7F is the value that is used in calculating the TCP value for the toxoid. All other rows are disregarded and not used in the TCP value calculation.

As the 500 µL reaction mixture, in the end-point well, contains 125 µL toxoid, the final dilution factor of the toxoid in the reaction mixture is the initial dilution (applied when preparing each dilution step) multiplied by 4. The final concentration of the antitoxin in the ternary equilibrium complex is 0.2 IU/mL half of which (0.1 IU/mL) is bound by the toxoid and the other half (0.1 IU/mL) is bound by the detector toxin in the end-point well. The TCP value refers to the equilibrium between the toxoid and 1 IU/mL antitoxin, therefore the final dilution at the end-point (4 × the end-point dilution value) of the toxoid has to be normalised to 1 IU/mL antitoxin. Thus, the TCP is calculated by dividing the final dilution value at the end-point by 10 (i.e. the TCP value is practically the end-point dilution value multiplied by 0.4).

ANNEX 6: STATISTICAL METHODS

Assay results were combined to generate unweighted geometric mean results (GM) for each laboratory and these means were used to calculate overall unweighted geometric mean results (Overall GM). Variability between assays and participants was expressed using geometric coefficients of variation (GCV = {10S-1} × 100 % where S is the standard deviation of the log10 transformed results). These calculations were applied to the assay results related to the determination of the sensitivity of Vero cells, latent toxicity of test materials and TNE+ of the detector toxin and toxins for which log-normal distributions were considered. TCP results, for which a normal distribution was considered, were summarised using simple means (AM) and coefficients of variation (CV = S/AM where S is the standard deviation of the results).

ANNEX 7: INDIVIDUAL END-POINTS FROM VERO CELL SENSITIVITY ASSAYS

Labora-tory	Wave-length	EP #1	EP #2	EP #3	EP #4	EP #5	EP #6	Final EP	Median Blank	CV Neg. Ctrl	Median Neg. Ctrl	Cut-off	Comments
1	590	256	256	256	256	256	256	256	0.02	8	0.71	0.35	
2	570	512	512	512	512	512	512	512	0.06	14	3.61	1.81	
3	570	512	512	512	512	512	512	512	0.15	4	1.86	0.93	
4	630	256	256	256	256	256	256	256	0.03	13	0.45	0.23	
4	630	256	256	256	256	256	256	256	0.02	8	0.54	0.27	
5	570	64	64	64	64	64	32	64	0.12	6	3.27	1.64	
6	630	256	256	256	256	256	256	256	0.06	11	0.45	0.22	
7	630	512	512	512	512	512	512	512	0.01	7	0.58	0.29	
8	570	512	512	512	512	512	512	512	0.01	5	0.24	0.12	
9	560	1024	1024	1024	1024	1024	1024	1024	0.07	7	1.14	0.57	Vero 2B20 – Selected
10	620	1024	1024	1024	1024	1024	512	1024	0.01	6	0.59	0.30	
11	620	1024	1024	1024	1024	1024	1024	1024	0.01	12	0.31	0.15	
12	570	128	128	128	128	128	128	128	0.16	10	1.14	0.57	
13	630	512	512	512	256	256	512	512	0.06	14	0.72	0.36	
14	570	128	128	128	128	128	128	128	0.03	8	1.11	0.55	Assay 1
14	570	64	64	64	128	64	128	64	0.03	7	0.96	0.48	Assay 2
Extra (not analysed)													
2	630	512	512	512	512	512	512	512	0.01	17	0.64	0.32	Reading 630 nm
9	560	1024	1024	1024	1024	SEQ	SEQ	SEQ	0.13	13	1.01	0.51	Vero G
9	560	1024	1024	1024	1024	1024	1024	1024	0.10	8	1.08	0.54	Vero L
12	630	128	128	128	128	128	128	128	0.02	13	0.50	0.25	Reading 630 nm

EP: end-point reported as inverse dilution/1000. SEQ: invalid end-point (positive results did not happen in consecutive wells).

ANNEX 8: INDIVIDUAL END-POINTS FROM LATENT TOXICITY ASSAYS

Laboratory	Wavelength	TdA	TdC	TdD	TdN	TdO	TdP	Antitoxin	Median Blank	CV Neg. Ctrl	Median Neg. Ctrl	Cut-off
1	590	40	(80)	20	LOW	320	40	LOW	0.03	11	0.67	0.34
2	570	20	80	5	LOW	320	10	LOW	0.04	9	3.15	1.57
3	570	160	20	5	LOW	640	160	LOW	0.14	7	1.56	0.78
4	630	10	80	10	LOW	160	10	LOW	0.02	26 (Inv.)	0.32	0.16
4	630	SEQ	SEQ	SEQ	SEQ	SEQ	10	LOW	0.02	20	0.33	0.17
4	630	10	80	10	LOW	80	20	LOW	0.02	9	0.53	0.27
5	570	10	80	10	LOW	320	LOW	LOW	0.11	13	3.25	1.62
6	630	20	80	10	LOW	320	40	LOW	0.01	17	0.42	0.21
7	630	40	40	5	LOW	320	40	LOW	0.00	15	0.51	0.25
8	570	160	(20)	5	LOW	(640)	(160)	LOW	0.02	7	2.08	1.04
9	560	80	80	5	LOW	640	160	LOW	0.04	16	1.06	0.53
10	540	40	80	5	LOW	640	80	LOW	0.02	4	0.68	0.34
10	540	40	40	5	LOW	320	80	LOW	0.02	8	0.66	0.33
11	620	40	160	20	LOW	320	80	LOW	0.01	17	0.29	0.15
12	570	10	40	5	LOW	80	10	LOW	0.19	5	2.65	1.33
13	630	10	(40)	LOW	5	(80)	SEQ	LOW	0.06	9	0.74	0.37
13	630	20	(40)	LOW	5	SEQ	SEQ	LOW	0.07	5	0.86	0.43
13	630	NP	20-40	NP	NP	(160)-160	80-80	LOW	0.07	13	0.44	0.22
14	570	40	40	LOW	LOW	80	(20)	LOW	0.04	9	0.99	0.50
14	570	40	40	5	LOW	80	20	LOW	0.04	12	1.05	0.53
Extra (not analysed)												
2	630	20	80	5	LOW	320	10	LOW	0.00	10	0.52	0.26
12	630	10	40	5	LOW	80	10	LOW	0.02	8	1.10	0.55

Results as inverse dilutions for toxoids, in IU/mL for the Standard Antitoxin. SEQ: invalid end-point (positive results did not happen in consecutive wells).
(Inv.): invalid assay (CV > 20 %). NP: not performed.

Notes

Some participants experienced a so-called “hook effect” for one or more toxoids, i.e. a negative result in the first well followed by positive results in the next wells then further negative wells. Such an effect may be caused by a supposed protective effect of the matrix at low dilution. As such effects are not unusual in bioassays, the first negative wells were disregarded, and the corresponding end-points were accepted as valid, despite the fact that they did not meet the validity criterion: “negative and positive wells must be in consecutive positions”. The results in these cases are reported in brackets and are taken into account in the statistical analysis.

Laboratory 10 reported the results of 2 plates in the same assay.

Laboratories 2 and 12 read the plates using two wavelengths (570 and 630 nm).

Laboratory 13 tested TdC, TdO and TdP in duplicate rows in the third assay.

ANNEXES 9A AND B: INTERMEDIATE END-POINTS FROM TNE+ ASSAYS OF THE DETECTOR TOXIN (CSTX2) AT 1.3-FOLD DILUTIONS

ANNEX 9A. INITIAL ASSAY (1.3-FOLD DILUTIONS), PLATE 1

Labora-tory	Wave-length	Min	Max	Inter. end-point	Median blank	CV Neg. Ctrl	Median Neg. Ctrl	Cut-off
1	590	887.25	887.25	887.25	0.06	6	1.42	0.71
2	570	887.25	887.25	887.25	0.07	3	3.49	1.75
3	570	887.25	887.25	887.25	0.17	5	1.89	0.95
4	630	887.25	887.25	887.25	0.06	7	0.34	0.17
4	630	887.25	887.25	887.25	0.02	13	0.51	0.26
4	630	887.25	887.25	887.25	0.01	5	0.56	0.28
5	570	1 153.425	1 153.425	1 153.425	0.15	5	3.28	1.64
6	630	887.25	887.25	887.25	0.03	12	0.48	0.24
7	630	887.25	887.25	887.25	0.00	18	0.41	0.21
8	570	1 153.425	1 153.425	1 153.425	0.03	8	2.34	1.17
9	560	1 499.4525	1 499.4525	1 499.4525	0.07	17	0.80	0.40
10	620	1 499.4525	1 499.4525	1 499.4525	0.02	5	0.34	0.17
11	620	887.25	887.25	887.25	0.02	52 (Inv.)	0.31	0.16
11	620	1 153.425	1 153.425	1 153.425	0.01	72 (Inv.)	0.08	0.04
11	620	1 153.425	1 153.425	1 153.425	0.04	6	1.35	0.68
12	570	1 153.425	1 153.425	1 153.425	0.07	11	2.45	1.22
13	630	1 153.425	1 153.425	1 153.425	0.04	17	0.57	0.28
14	570	1 153.425	1 153.425	1 153.425	0.03	12	0.84	0.42
14	570	887.25	1 153.425	887.25	0.04	10	1.11	0.55
14	570	1 153.425	1 153.425	1 153.425	0.05	6	1.18	0.59

Min & Max: minimum and maximum individual end-point (inverse dilution) among 6. (Inv.): invalid assay (CV > 20 %).

ANNEX 9B. INITIAL ASSAY (1.3-FOLD DILUTIONS), PLATE 2

Labora-tory	Wave-length	Min	Max	Inter. end-point	Median blank	CV Neg. Ctrl	Median Neg. Ctrl	Cut-off
1	590	887.25	887.25	887.25	0.10	8	1.26	0.63
2	570	887.25	887.25	887.25	0.07	3	3.58	1.79
3	570	887.25	887.25	887.25	0.12	6	1.44	0.72
4	630	525	682.5	525	0.05	9	0.31	0.15
4	630	887.25	887.25	887.25	0.02	7	0.48	0.24
4	630	887.25	887.25	887.25	0.01	11	0.54	0.27
5	570	1 153.425	1 153.425	1 153.425	0.11	6	3.22	1.61
6	630	887.25	887.25	887.25	0.02	10	0.48	0.24
7	630	887.25	887.25	887.25	0.00	17	0.51	0.25
8	570	1 153.425	1 153.425	1 153.425	0.03	5	2.53	1.26
9	560	1 499.4525	1 499.4525	1 499.4525	0.08	16	0.38	0.19
10	620	1 499.4525	1 499.4525	1 499.4525	0.02	8	0.38	0.19
11	620	887.25	SEQ	SEQ	0.02	8	0.86	0.43
11	620	1 153.425	SEQ	SEQ	0.02	8	0.69	0.34
11	620	1 153.425	1 153.425	1 153.425	0.03	3	1.21	0.61
12	570	1 153.425	1 153.425	1 153.425	0.07	6	2.68	1.34
13	630	1 153.425	1 153.425	1 153.425	0.05	19	0.46	0.23
14	570	1 153.425	1 153.425	1 153.425	0.04	27 (Inv.)	0.82	0.41
14	570	887.25	1 153.425	887.25	0.04	9	0.99	0.50
14	570	1 153.425	1 153.425	1 153.425	0.05	13	0.96	0.48

Min & Max: minimum and maximum individual end-point (inverse dilution) among 6. (Inv.): invalid assay (CV > 20 %). SEQ: invalid end-point (positive results did not occur in consecutive wells).

ANNEX 9C TO E: INTERMEDIATE END-POINTS FROM TNE+ ASSAYS OF THE DETECTOR TOXIN (CSTX2) AT 1.1-FOLD DILUTIONS

ANNEX 9C. FIRST ASSAY (1.1-FOLD DILUTIONS)

Labora-tory	Wave-length	Interm. end-points		Median blank		CV Neg. Ctrl		Cut-off	
		P1	P2	P1	P2	P1	P2	P1	P2
1	590	245	261	0.12	0.31	5	7	0.44	0.40
2	570	315	315	0.03	0.04	14	3	1.58	1.76
3	570	287	287	0.12	0.14	5	5	0.81	0.87
4	630	237	(245)	0.02	0.02	37 (Inv.)	12	0.08	0.18
4	630	252	245	0.01	0.01	19	17	0.14	0.22
5	570	230	230	0.30	0.28	10	5	1.15	1.45
6	630	SEQ	SEQ	0.05	0.04	12	14	0.20	0.17
6	630	237	237	0.01	0.01	17	14	0.17	0.19
7	630	230	233	0.01	0.01	5	11	0.37	0.39
8	570	306	306	0.03	0.05	20	30 (Inv.)	0.87	0.96
8	570	278	278	0.01	0.01	10	8	0.85	0.79
9	560	439	439	0.03	0.04	12	13	0.27	0.24
10	540	330	330	0.02	0.02	2	3	0.36	0.41
11	620	348	348	0.03	0.03	13	11	0.40	0.55
12	570	342	326	0.05	0.06	3	3	1.63	1.41
13	630	371	371	0.09	0.06	10	6	0.29	0.28
14	570	(261)	SEQ	0.03	0.04	17	9	0.48	0.47
14	570	287	287	0.03	0.04	8	12	0.57	0.63
14	570	306	306	0.05	0.07	8	11	0.41	0.41

Interm. end-points P1 and P2: intermediate end-points (IU/mL) of plates 1 and 2. (Inv.): invalid assay (CV > 20 %). Bracketed end-points: calculated following exclusion of one false negative well. SEQ: invalid end-point (positive results did not happen in consecutive wells).

ANNEX 9D. SECOND ASSAY (1.1-FOLD DILUTIONS)

Labora-tory	Wave-length	Interm. end-points		Median blank		CV Neg. Ctrl		Cut-off	
		P1	P2	P1	P2	P1	P2	P1	P2
1	590	215	215	0.05	0.15	10	19	0.73	0.62
2	570	315	315	0.06	0.07	5	3	1.47	1.67
3	570	261	261	0.10	0.10	6	5	0.73	0.78
4	630	237	241	0.02	0.02	13	12	0.27	0.27
5	570	253	253	0.17	0.18	13	11	1.27	1.50
6	630	(237)	(233)	0.23	0.27	14	16	0.12	0.13
6	630	261	261	0.01	0.01	14	17	0.17	0.17
7	630	237	233	0.00	0.00	7	8	0.35	0.37
8	570	297	297	0.03	0.02	17	15	0.48	0.46
9	560	363	363	0.04	0.05	10	12	0.42	0.47
10	540	330	325	0.02	0.03	5	3	0.37	0.37
11	620	382	376	0.03	0.02	9	3	0.38	0.49
12	570	337	321	0.04	0.06	7	15	0.83	0.68
13	630	371	366	0.07	0.09	10	12	0.27	0.31
14	570	261	261	0.03	0.04	11	20	0.61	0.48
14	570	278	278	0.07	0.06	16	12	0.40	0.40

Interm. end-points P1 and P2: intermediate end-points (IU/mL) of plates 1 and 2. Bracketed end-points: calculated following exclusion of one false negative well.

ANNEX 9E. THIRD ASSAY (1.1-FOLD DILUTIONS)

Laboratory	Wavelength	Interm. end-points		Median blank		CV Neg. Ctrl		Cut-off	
		P1	P2	P1	P2	P1	P2	P1	P2
1	590	237	237	0.04	0.05	7	4	0.41	0.41
2	570	315	315	0.06	0.05	2	2	1.87	1.90
3	570	265	269	0.25	0.17	6	9	1.06	1.18
4	630	261	261	0.02	0.02	9	5	0.27	0.27
5	570	230	230	0.18	0.16	10	17	1.22	1.41
6	630	230	226	0.01	0.01	15	19	0.27	0.19
7	630	215	215	0.00	0.00	9	8	0.39	0.42
8	570	306	306	0.04	0.04	10	12	1.04	1.15
9	560	412	432	0.05	0.05	11	17	0.25	0.25
10	540	330	320	0.03	0.03	4	4	0.43	0.41
11	620	370	370	0.03	0.03	7	4	0.43	0.47
12	570	297	301	0.07	0.07	9	7	0.74	0.70
13	630	338	371	0.04	0.05	15	15	0.32	0.33
14	570	261	261	0.03	0.05	6	14	0.67	0.74
14	570	306	306	0.07	0.06	12	14	0.41	0.52

Interm. end-points P1 and P2: intermediate end-points (IU/mL) of plates 1 and 2.

ANNEX 10: FINAL END-POINTS FROM TNE+ EVALUATION OF THE TEST TOXINS IN THE 1.3-FOLD DILUTION ASSAYS

Laboratory	End-points	GM	GCV	End-points	GM	GCV	End-points	GM	GCV
1	5, 5, 9	6	41	14, 14, 23	16	35	14, 14, 18	15	16
2	14, 14, 14	14	0	27, 27, 24	26	8	23, 23, 23	23	0
3	10, 10, 14	11	16	21, 21, 27	23	16	18, 18, 23	19	16
4	20, 18, 15	18	14	35, 27, 29	30	15	31, 33, 35	33	7
5	18, 18, 20	18	8	26, 23, 30	26	14	52, 56, 35	47	28
6	11, 11, 9	10	16	18, 14, 14	15	16	14, 16, 14	14	12
7	14, 18, 10	14	30	22, 27, 21	23	15	29, 25, 21	25	18
8	16, 16, 16	16	0	27, 27, 27	27	0	27, 27, 27, 27	27	0
9	18, 14, 14	15	15	35, 27, 27	30	16	27, 27, 27	27	0
10	16, 14, 14	14	12	35, 35, 35	35	0	31, 35, 35	34	8
11	29, 21, 21, 16	21	27	38, 35, 27, 27	31	19	35, 27, 22	28	26
12	23, 23, 20	22	8	35, 46, 35	38	16	30, 30, 34	31	8
13	39, 30, 39	35	16	46, 46, 46	46	0	59	59	–
	TxR	**15**	**51**	**TxS**	**27**	**37**	**TxV**	**27**	**49**

Laboratory	End-points	GM	GCV	End-points	GM	GCV	End-points	GM	GCV
1	108, 108, 141	118	16	141, 141, 183	153	16	54, 54, 70	59	16
2	160, 183, 183	175	8	216, 281, 281	258	16	91, 91, 91	91	0
3	141, 141, 183	153	16	216, 166, 216	198	16	70, 70, 75	72	4
4	141, 150, 183	157	15	216, 231, 281	241	15	86, 91, 91	89	4
5	131, 131, 171	143	16	150, 183, 183	171	12	111, 91, 111	104	12
6	141, 141, 141	141	0	183, 141, 141	153	16	70, 54, 54	59	16
7	183, 223, 141	179	26	216, 247, 231, 216	227	6	91, 91, 91, 91	91	0
8	216, 166, 166, 166	178	14	333, 216, 216, 216	241	24	108, 95, 83, 83	92	13
9	141, 141, 141	141	0	216, 216, 216	216	0	78, 78, 78	78	0
10	183, 183, 183	183	0	216, 281, 216	236	16	91, 91, 91	91	0
11	183, 183, 223	195	12	281, 281, 281	281	0	108, 108, 108	108	0
12	183, 183, 183	183	0	247, 281, 263	263	7	80, 119, 104	100	22
13	238	238	–	(366), 366	366	0	(91), 75	83	15
	TxW	**165**	**20**	**TxY**	**225**	**28**	**TxZ**	**85**	**21**

ANNEX 11: FINAL END-POINTS (TCP UNITS) FROM THE TCP EVALUATION OF THE TEST TOXOIDS IN THE 10-INCREMENT DILUTION ASSAYS

Laboratory	End-points	AM	CV	End-points	AM	CV	End-points	AM	CV
2	68, 68, 56	64	11	6, 6, 8	7	17	28, 24, 28	27	9
3	68, 62, 64, 72	67	7	4, 4	4	0	26, 16, 24	22	24
4	68, 66, 70	68	3	10, 10, 8	9	12	20, 32, 20	24	29
5	76, 76, 76	76	0	18, 18, 14	17	14	64, 66, 64	65	2
6	72, 68, 64	68	6	4, 4, 4	4	0	28, 28, 30	29	4
7	–	–	–	8	8	–	38	38	–
8	76, 76, 68	73	6	8, 6, 14	9	45	40, 32, 38	37	11
9	84, 100, 100, 88	93	9	8, 8, 8	8	0	32, 24, 42	33	28
10	80, 88, 92	87	7	4, 4, 4	4	0	36, 30, 32	33	9
11	80, 80, 76	79	3	4, 8, 6	6	33	28, 28, 28	28	0
12	84, 90, 76	83	8	4, 6, 4	5	25	24, 18, 8	17	48
	TdA	**76**	**13**	**TdC**	**7**	**51**	**TdD**	**32**	**39**

Laboratory	End-points	AM	CV	End-points	AM	CV	End-points	AM	CV
2	12, 12, 12	12	0	112, 124, 128	121	7	92, 112, 112, (96)	103	10
3	8, 12, 8, 12	10	23	(86), 96, (94), 112, 88	95	11	78, 86, 82	82	5
4	12, 12, 12	12	0	100, 94, 100	98	4	90, 90, 80	87	7
5	40, 42, 42	41	3	92, 92, 92	92	0	108, 108, 108	108	0
6	12, 12, 16	13	17	100, 100, 108	103	4	84, 84, 84	84	0
7	12	12	–	–	–	–	–	–	–
8	16, 14, 14	15	8	112, 112, 120	115	4	116, 114, 108	113	4
9	18, 12, 28	19	42	132, 148, 148	143	6	120, 130, 132, (120)	126	5
10	20, 22, 20	21	6	142, 124, 132	133	7	132, 118, 130	127	6
11	16, 16, 20	17	13	108, 108, 120	112	6	100, 120, 120	113	10
12	12, 20, 8	13	46	102, 116, 112	110	7	100, 120, 116	112	9
	TdN	**17**	**52**	**TdO**	**112**	**15**	**TdP**	**105**	**15**

Bracketed end-points: calculated following exclusion of one false negative well.