Collaborative study for the validation of cell line assays for in-process toxicity and antigenicity testing of *Clostridium septicum* vaccine antigens – Part 1

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ABSTRACT

Large numbers of mice are used in testing during the production of Clostridial vaccines. Previous work has indicated that cell line assays could replace mouse tests for certain aspects of this testing. Replacement assays have been developed for the testing of the toxins and toxoids of several clostridial species but none of these assays have 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), collaborative study BSP130 was initiated to evaluate Vero cell based alternative methods to the current mouse tests used to measure the toxicity of Clostridium septicum toxin (the minimum lethal dose (MLD) test), the freedom from toxicity of C. septicum toxoid (the MLD test) and the antigenicity of C. septicum toxoid (the total combining power (TCP) test). The principal aims of BSP130 were to determine the repeatability and reproducibility of the in vitro assays and to demonstrate concordance of the proposed in vitro and current in vivo TCP and MLD tests. 11 laboratories from 7 countries participated in the collaborative study and each tested 6 toxins and 6 toxoids. The participants' Vero cell lines were up to 1000 times more sensitive than the mouse strains. The MLD assay in mice and on Vero cells generally ranked the toxins in a similar order in most of the laboratories. The TCP assay in mice and on Vero cells also generally ranked the toxoids in a similar order in most of the laboratories. The results demonstrate that the repeatability and reproducibility of the in vitro Vero cell based assays are no worse than that of the in vivo assays and that they are easily transferable to other laboratories. The concordance correlations between the in vivo and in vitro methods were for the MLD assays $\rho_c = 0.961$ (log-transformed values) and $\rho_c = 0.921$ (non-log-transformed values) and for the TCP assays $\rho_c = 0.968$ (log-transformed values) and $\rho_c = 0.980$ (non log-transformed values). These correlations are excellent showing that the Vero cell assays can be used as alternatives to the mouse tests for the assessment of C. septicum toxin MLD and toxoid TCP values. This study can be used by vaccine manufacturing companies as a guide for applying the same approach to other clostridial toxins and toxoids.

KEYWORDS

Clostridium septicum vaccine, minimum lethal dose, residual toxicity, total combining power, European Partnership for Alternative Approaches to Animal Testing, Biological Standardisation Programme, EDQM.

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1. INTRODUCTION

1.1. Background information on 3Rs

In view of the expectations of the 3Rs: replacement, reduction and refinement of animal assays as proposed by Russell and Burch in 1959, the Council of Europe, a pioneer in the field of 3Rs, created in 1986 the first legally binding European instrument by opening for signature the international European Treaty (ETS No. 123), European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes 1. Based on this convention, the European Union (EU) adopted in November 1986 Directive 86/609/EEC *Animals used for scientific purposes*, subsequently replaced by Directive 2010/63/EU, which came into effect on 1 January 2013.

In line with the Council of Europe and EU policy, the European Pharmacopoeia (Ph. Eur.), supported by its secretariat, EDQM, has been involved actively in the replacement, reduction and refinement of animal assays.

Beside the pharmacopoeia activities, the Official Medicines Control Laboratory (OMCL) network – in particular, the network for Official Control Authority Batch Release (OCABR) for human and veterinary biologicals 2 and the Biological Standardisation Programme (BSP) 3 actively works to improve the implementation of the 3R approaches in the control of the pharmaceutical quality of medicines 4.

In the field of vaccines 5 the Ph. Eur. Commission is continuing its efforts to reduce the number of animals needed to perform tests 6 e.g. through harmonisation of all the veterinary vaccine monographs and by continuous revision of general texts and monographs to re-evaluate the relevance of animal tests mentioned in European Pharmacopoeia texts (lately, in the interest of the 3Rs, the Ph. Eur. Commission also adopted the deletion of the target animal batch safety test for all veterinary vaccines) and, if deemed appropriate, to include alternative methods.

The BSP, a research programme aimed at validating new pharmacopoeia methods and establishing Ph. Eur. reference preparations, is particularly committed to considering promising alternative 3Rs methods.

The EU, notably through its Reference laboratory for alternatives to animal testing (EURL-ECVAM 7, JRC 8) is also committed to address the 3Rs issues remaining in the field of quality control of medicines. In a recent initiative, the European Commission (EC) joined forces with the pharmaceutical industry by creating the European Partnership for Alternative Approaches to Animal Testing (EPAA), a voluntary collaboration between the EC, European trade associations and companies from seven industry sectors 9. As a member of the Vaccines Project Team, the EDQM contributes to the technical platform for 3Rs in regulation of the EPAA.

1.2. Background information on the project

In Europe, production and quality control of human and veterinary medicines account for the use of large numbers of animals which represent about 14 % of the total number of animals used for scientific purposes 10.

Under the aegis of the EPAA project ‘Application of the 3Rs and Consistency Approach for Improved Vaccine Quality Control’, the quality control methods for several vaccine categories were considered from the 3Rs perspective and potential improvement possibilities were evaluated. As it is commonly recognised that a large number of animals are currently used in the toxicity and antigenicity testing of clostridial vaccines 11, work in this field was given the highest priority.

Clostridial toxoid vaccine antigens are based on a number of organisms including: *Clostridium perfringens* type A, B, C and D, *C. novyi* type B, *C. septicum*, *C. haemolyticum, C. sordelli, C. difficile, C. tetani, C. botulinum* and *C. chauvoei.*

These vaccines are produced based on a common, simplified toxoid vaccine manufacturing process 12 consisting of the following steps:

1. growth of the organism
2. removal of cells (centrifugation and/or filtration)
3. chemical inactivation of toxins in supernatants
4. blending with other antigens and adjuvant
5. dispensation in vaccine.

Analytical procedures are undertaken at each step and several tests are based on *in vivo* tests: in process tests (toxicity of toxin, residual toxicity of toxoid and antigenicity of toxoid) and batch potency testing. These tests account for the use of large numbers of animals 12.

For batch potency testing of clostridial vaccines, serological tests and corresponding references have been proposed and included in the Ph. Eur. monographs based on BSP studies 13, 14, 15 or studies by others 16, 17.

The EPAA experts group on the Application of the 3Rs and Consistency Approach for Improved Vaccine Quality Control evaluated a preliminary investigative work, supported by a National Centre for the Replacement Refinement & Reduction of Animals in Research (NC3Rs) grant, which indicated that a possible alternative would be to develop cell line assays to replace mouse based assays for certain aspects of the in-process control testing of various clostridial toxoid vaccine antigens sharing the feature of being produced by inactivation of a major cytotoxin (*C. perfringens* type A, B, C and D, *C. novyi* type B, *C. septicum*, *C. haemolyticum, C. sordelli* and *C. difficile*). Under the NC3Rs grant, replacement *in vitro* assays were developed for the MLD and TCP testing of the toxins and toxoids of several clostridial species 18. However, the evaluation of all of these *in vitro* assays would have required time and resources well beyond the scope of a typical collaborative study. It was therefore decided to initially evaluate the *in vitro* assays for the toxin and toxoid of just one clostridial species.

The species chosen for this study was *Clostridium septicum* on the following basis: it is commonly a component of veterinary combination clostridial vaccines and *in vitro* toxicity (also referred to as minimum lethal dose) and antigenicity (also referred to as total combining power) assays have been developed for this species and it is the widely available Vero cell line that is used. It was also expected that potential participants in the collaborative study would have experience in the *in vivo* testing of this toxin and toxoid according to the Ph. Eur. monograph *Clostridium septicum vaccine for veterinary use* (0364).

In March 2013, the members of the EPAA project Application of the 3Rs and Consistency Approach for Improved Vaccine Quality Control approved the start and invited the EDQM, an active member in the EPAA process, to co-sponsor and co-ordinate the proposed study 19. If successful the study would support the concept of using an alternative (cell line) to the mouse model as toxicity indicator for clostridial vaccines in-process testing.

After its formal approval by the BSP steering committee, the study was initiated under the aegis of the EDQM Biological Standardisation Programme, as project BSP130, with the full support of EPAA, who provided human and financial resources.

Dr Keith Redhead and Dr Lukas Bruckner were nominated as project leaders and 11 laboratories committed to participate.

2. AIMS OF THE STUDY

The collaborative study aimed at evaluating the transferability and the performances of alternative methods to the current *in vivo* mouse tests used to measure the toxicity of C. septicum toxin (the minimum lethal dose (MLD) test), the freedom from toxicity of *C. septicum* toxoid (the MLD test) and the antigenicity of C. septicum toxoid (the total combining power (TCP) test)(see definitions in Appendix 1-2. Terminology and definitions).

The principal aims of BSP130 were to demonstrate the correlation of proposed *in vitro* and current compendial *in vivo* TCP and MLD tests as described in the Ph. Eur. monograph *Clostridium septicum vaccine for veterinary use (0364, Ph. Eur. 8th Ed.)* (Appendices 1-1 and

1-2) and to determine the repeatability and reproducibility of the *in vitro* assays using data obtained from the laboratories of the participants in the collaborative study.

The replacement *in vitro* assays were expected to be basically the same as the *in vivo* tests except that after the toxin dilutions necessary for the MLD and the toxoid, antitoxin and toxin mixing and reactions necessary for the TCP the final materials are assessed for indications of toxicity not in mice but on a cell line.

The reliability of the Vero cell assays and of the mouse tests were to be studied by:

- obtaining information on intra-laboratory variation (inter-assay precision and repeatability).
- obtaining information on inter-laboratory variation (reproducibility).

The relationship between the Vero cell assays and the mouse tests were to be studied by looking for concordance between the relevant *in vivo* and *in vitro* assays.

In phase I, the proposed study samples (toxoid and toxins) were centrally collected by Dr Redhead and prequalified at MSD Animal Health UK by *in vivo* and *in vitro* methods (Appendix 2). Specifications of the material were included in the study protocol.

In phase II (collaborative study), to confirm the appropriateness of the test methods and reagents and to obtain preliminary ranges for the values of the test toxins and toxoids, the study was divided into four consecutive steps.

- Step 1: confirmation of sensitivity of mouse strains and cell lines.
- Step 2: latent toxicity testing of test materials.
- Step 3: preliminary ranging of test materials.
- Step 4: full testing of test materials.

3. PARTICIPANTS

11 laboratories from 7 countries participated in the collaborative study including 5 public laboratories (Official Medicines Control Laboratories (OMCLs) and other public institutions) and 6 manufacturers. Two laboratories which enrolled initially were unable to provide study results due to lack of human resources.

The participants are listed alphabetically in section 9 of this report.

4. MATERIAL, METHODS AND STUDY DESIGN

4.1. Material

4.1.1. C. septicum toxins

Six batches of *C. septicum* toxin (samples coded names TxA to TxF) obtained from various manufacturers and production sites and of differing toxicities, were used in the study. Details of their approximate toxicities (MLD in mice and Vero cells) are supplied in Appendix 2. The samples were supplied frozen on dry-ice.

4.1.2. C. septicum toxoids

Six batches of C. septicum toxoid (samples coded names TdG to TdM) obtained from various manufacturers and production sites and of differing antigenicities, were used in the study. Details of their approximate antigenicities (TCP in mice and Vero cells) are supplied in Appendix 2. The samples were supplied at + 2 to + 8 °C.

4.1.3. Standards and critical reference reagents

Standard antitoxin

Clostridium septicum (gas gangrene) antitoxin (coded name VI), equine, 3rd International Standard (IS) with defined activity of 500 IU/ampoule. The antitoxin was supplied as a freeze-dried powder at + 2 to + 8 °C.

Reference/detecting toxin

Clostridium septicum reference/detecting toxin (coded name CSTx), approximate L^+ value (see definition in Appendix 1-2. Terminology and definitions) 1/170 mL. The toxin was supplied frozen on dry-ice.

4.1.4. Storage conditions and use of test samples and references

Toxins

All test and reference toxins were delivered as vials containing sterile frozen aliquots of approximately 1 mL each.

The toxins were to be initially stored frozen at less than − 15 °C. When ready for testing, one 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 vials were to spend the minimum amount of time at temperatures above + 8 °C. When a toxin aliquot had been thawed but only a portion of it had been used, provided it was still sterile, the rest of the toxin could be stored at + 2 to + 8 °C for up to four weeks for further use.

Toxoids

All test toxoids were delivered as bijous containing sterile chilled (+ 2 to + 8 °C) aliquots of approximately 3 mL each.

The toxoids were to be stored at + 2 to + 8 °C prior to use. All manipulations of the toxoids were to be performed under sterile conditions and the toxoid bijous were to spend the minimum amount of time at temperatures above + 8 °C. Once opened a bijou of toxoid, provided it was still sterile, could remain stored at + 2 to + 8 °C for further use.

***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 below − 15 °C until needed.

For *in vivo* TCP assays, and the CSTx L^+ determination where performed, thawed 1.0 mL aliquots of *C. septicum* standard antitoxin (VI) were to be diluted and used according to the relevant laboratory's own methodologies. Details of the antitoxin dilutions used were to be entered in the remarks section of the participant's *in vivo* TCP information and in the provided reporting sheet.

For use by laboratories performing the *in vitro* only TCP assays, thawed 1.0 mL aliquots of the antitoxin were to be 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 to be retained for use in the detecting toxin (CSTx) determination. To the remaining 7.0 mL of the solution was to be added 1.75 mL of sterile NBS to give 8.75 mL of 4 IU/mL for use in the *in vitro* TCP determinations. If there were any variations from this approach the details were to be entered in the comments section of the appropriate electronic reporting sheet.

Additional information, including specifications, codes and quantities of material provided can be found in Appendix 2.

4.2. Methods

The methods used in BSP130 were *in vivo* MLD assay in mice, *in vitro* Vero cell MLD assay, *in vivo* TCP assay in mice and *in vitro* Vero cell TCP assay (Appendix 1-1). *In vivo* tests were performed using the in-house routine methods and *in vitro* tests were performed using the standard operating procedures (SOP) given in the study protocol and according to the principles described below.

In vivo MLD assay in mice was performed using the method routinely employed within the participant's laboratory. A copy of the methodology or SOP was shared with the project leaders. For each test toxin the result obtained from the preliminary ranging test was used as the central value in a range of 5 3-fold dilutions which stretch to 2 dilutions above and below that value. If the 3-fold dilution series was found to give inconsistent results, an appropriate 5-fold dilution series was used. Each of the 5 dilutions was assessed in a pair of mice, which were monitored for lethal effects of the toxin. The aim was to report the results of 3 valid assays; however, the results from all of the assays performed were requested.

In vivo TCP assay in mice was performed using the method routinely employed within the participant's laboratory. A copy of the methodology or SOP was shared with the project leaders. For each test toxoid the result obtained from the preliminary ranging test was used as the central value in a series of 5 dilutions which increase by no more than 20 TCP units per dilution. Each of the 5 dilutions was assessed in a pair of mice, which were monitored for lethal effects of the toxin. The aim was to report the results of 3 valid assays; however, the results from all of the assays performed were requested.

In vitro Vero cell MLD assay was performed according to the methodology provided in the study protocol. For each test toxin the result obtained from the preliminary ranging test was used as the central value in a range of 5 3-fold dilutions which stretch to 2 dilutions above and below that value. If the 3-fold dilution series was found to give inconsistent results an appropriate 5-fold dilution series was to be used. Each of the 5 dilutions is assessed in 2 rows of Vero cells for lethal effects of the toxin. The aim was to report the results of 3 valid assays; however, the results from all of the assays performed were requested.

In vitro Vero cell TCP assay was performed according to the methodology provided in the study protocol. For each test toxoid the result obtained from the preliminary ranging test was to be used as the central value in a series of 5 dilutions which increase by no more than 20 TCP units per dilution. Each of the five dilutions was assessed in 2 rows of Vero cells for lethal effects of the toxin. The aim was to report the results of 3 valid assays; however, the results from all of the assays performed were requested.

5 laboratories performed both *in vitro* and *in vivo* tests, 5 laboratories performed only *in vitro* tests and 1 performed only *in vivo* tests. An overview of the methods performed by each laboratory is presented in Appendix 3 and methodological details as reported by participants are presented in Appendix 4.

4.3. Study design

In November 2013, each participating laboratory was provided with panels of samples comprising 6 test toxins (coded TxA, TxB, TxC, TxD, TxE and TxF and 6 test toxoids (coded TdG, TdH, TdJ, TdK, TdL and TdM), and with the standard antitoxin and the reference/detecting toxin (CSTx).

In vivo testing in mice was to be performed by those participants that already routinely performed this form of testing and, therefore, had their own methodologies for these tests. It was expected that these participants would use their in-house methods with the only modifications being the dilution values that were assessed. *In vitro* testing in the Vero cells based-assays was to be performed in accordance with the methodologies described in the study protocol. The methods performed by the participants were:

- *in vivo* MLD assay in mice, as performed within that laboratory with specified variations (provided in the study protocol);

- *in vivo* TCP assay in mice, as performed within that laboratory with specified variations (provided in the study protocol);
- *in vitro* Vero cell MLD assay, performed according to the methodology provided in the study protocol;
- *in vitro* Vero cell TCP assay, performed according to the methodology as provided in the study protocol.

The results of 3 valid assays for each assay type performed (see Appendix 3) were reported by each participant laboratory.

The experimental phase of the collaborative study was divided into 4 steps, to be run successively as described hereafter.

Step 1: confirmation of sensitivity of mouse strains and cell lines

The initial sensitivity of the mouse strains and Vero cell lines to *C. septicum* toxin was assessed in the *in vivo* and *in vitro* MLD tests, respectively, using CSTx. This toxin was subjected to a 5-fold dilution series from a concentration of 1 in 5 down to a concentration of 1 in 3 125. Each dilution was assessed in duplicate in a pair of mice and/or rows of Vero cells, as appropriate, which were then monitored for lethal effects of the toxin. The toxin was then subjected to a 3-fold dilution series from a suitable concentration above to a suitable concentration below the end-point determined in the 5-fold dilution series. Again each dilution was assessed in duplicate in a pair of mice and/or rows of Vero cells, as appropriate, which were then monitored for lethal effects of the toxin. If the 3-fold dilution series generated inconsistent results the toxin was to be re-assessed using an appropriate 5-fold dilution series.

From these results the participants determined an initial pre-dilution for the CSTx detecting toxin for use on the Vero cells that would result in the killing of the Vero cells for 4 to 6 doubling dilutions when applied to the plates. The CSTx was then used at this pre-dilution as the detecting toxin on all *in vitro* MLD Vero cell plates.

Step 2: latent toxicity testing of test materials

The standard *C. septicum* antitoxin (VI) was reconstituted, diluted and stored according to the instructions in the study protocol. It was then further diluted to a concentration of 5 IU/mL. Each of the 6 *C. septicum* test toxoids (TdG to TdM) was diluted 1 in 10. All 6 toxoids and the standard antitoxin were then tested, at these final concentrations, for toxicity in mice and/or Vero cells, as appropriate, using the relevant MLD method and the results reported.

Step 3: preliminary ranging of test materials

The preliminary ranging tests for all 6 test toxins were conducted in the *in vivo* and/or *in vitro*, as appropriate, MLD assays. Centred on the approximate MLD value supplied for each test toxin (Appendix 2), the participants were asked to perform a 5-step 5-fold dilution series ranging from approximately 25 times greater than the MLD value to 25 times less than the MLD value. Each of the 5 dilutions was assessed in a pair of mice and/or rows of Vero cells, as appropriate, which were then monitored for lethal effects of the toxin. When using the *in vitro* assay, participants were advised that if they found the Vero cells to be too sensitive to the lethal effects of some of the test toxins to give an end-point, at each step of the toxin dilution sequence a pre-dilution factor (as part of a 2-fold dilution sequence, i.e. 1 in 2, 1 in 4, 1 in 8, etc.) should be introduced prior to its doubling dilution and application to the Vero cell plate.

Prior to the range testing of the toxoids the participants running the *in vivo* TCP were requested to confirm the L^+ value for the CSTx detecting toxin in their *in vivo* test system. If the value they obtained was more than 10 % different from the supplied value they were asked to use their value in all subsequent *in vivo* TCP testing and for *in vitro* TCP testing if applicable. For participants performing *in vitro* only TCP testing the CSTx toxin was to be used at the supplied L^+ value.

The preliminary ranging tests for all 6 test toxoids were conducted in the *in vivo* and/or *in vitro*, as appropriate, TCP assays. Based on the approximate TCP value supplied for each test toxoid

(Appendix 2), the participants performed a dilution series ranging from approximately 80 TCP units less than the supplied TCP value, where appropriate, to approximately 80 TCP units above the TCP value. It was suggested that each step in the dilution sequence should differ by 40 TCP units, therefore requiring 5 dilutions to cover the full 160 unit range. Each of the 5 dilutions was assessed in a pair of mice and/or rows of Vero cells, as appropriate, which were then monitored for lethal effects of the toxin and the results recorded. As described above with the *in vitro* MLD assay, when using the *in vitro* TCP assay participants were advised that if they found that the Vero cells were too sensitive to the lethal effects of some of the test toxins to give an end-point, at each step of the toxin dilution sequence a pre-dilution factor (as part of a 2-fold dilution sequence, i.e. 1 in 2, 1 in 4, 1 in 8, etc.) should be introduced prior to its doubling dilution and application to the Vero cell plate and the pre-dilution factor recorded was to be reported.

Step 4: full testing of test materials

For the full collaborative study each of the test toxins and toxoids was tested, in the appropriate *in vivo* or *in vitro* assay. The testing was repeated on different days until a minimum of 3 valid assays had been completed for each test material in each test that was being assessed. All the results, including those from any invalid tests were reported. In the case of assays that were partially invalid only the materials for which invalid results were obtained needed to be subjected to repeat assays.

For participants performing both *in vivo* and *in vitro* assays, the result obtained from the *in vivo* preliminary ranging test was used as the central value in the range for the full *in vitro* testing of each relevant test sample.

For participants performing only the *in vitro* assays, the preliminary ranging test result that gave the end-point (the last well with greater than 50 % dead cells) closest to 5 doubling dilutions on the Vero plate was to be used as the central value in the range for the full *in vitro* testing of each relevant test sample. In this case, the value selected from the preliminary assay as the central value in the full testing range for each toxin or toxoid was to be reported.

5. RESULTS AND CENTRAL STATISTICAL ANALYSIS

11 laboratories reported results. A central statistical analysis of these results was performed at the EDQM. Due to the inherent novelty of the approach chosen, i.e. measure toxicity by assessing cytotoxicity on Vero cells instead of lethality in mice, a new approach to the statistical analysis of the results of MLD and TCP had to be developed and the resulting analysis and results produced follow. The statistical methods that were used to analyse the MLD and TCP individual assays are described in Appendix 5.

The MLD of *in vivo* assays was determined as the reciprocal of the last toxin dilution causing the death of both mice.

The TCP of *in vivo* assays was determined as the greatest toxoid dilution factor that (when reacted with the set amount of standard antitoxin) left insufficient antitoxin to fully neutralise the set amount of detector toxin resulting in the death of 1 mouse but not the other or as the arithmetic mean between the toxoid dilution factor that resulted in the death of both mice and the adjacent toxoid dilution factor that resulted in the survival of both mice.

Concordance between *in vivo* and *in vitro* assays was investigated by the use of 2-way plots and Lin's concordance correlation coefficient.

Step 1: confirmation of sensitivity of mouse strains and cell lines

Laboratories 1 to 6 reported results from sensitivity tests in mice and laboratories 2 to 10 reported results from sensitivity tests of Vero cell lines. For this purpose, the MLD of the common sample for *Clostridium septicum* toxin (CSTx) is used. The MLD is usually defined by the dilution containing the smallest amount of toxin still causing the death of both mice. This definition cannot be transferred in a straightforward way to the test on Vero cells because it is, a priori, not clear which endpoint should serve as a substitute for a dead mouse. Even if such an endpoint might be defined for an individual laboratory, it cannot be used across laboratories

since the endpoint depends on the sensitivity of mice as well as Vero cells. In addition, for statistical reasons it is difficult to work with endpoints in terms of 100 % lethality as it is biased by the number of replicates within the assay and can therefore not easily be extended to higher levels of replication. For these reasons, herein the MLD is defined as the dose of toxin causing 50 % lethality (LD50), corrected by half a dilution step in order to match the last dead experimental unit in the usual definition of the MLD. The sensitivity (S) of mice and Vero cells is defined as the LD50 of the detecting toxin (CSTx) expressed in the same units (LD50 in nL of CSTx per experimental unit or MLD in dilution of CSTx per experimental unit).

To illustrate this, consider the following example: 0.1 mL of 1/1 000 diluted CSTx was loaded into the first well of the first row of a microtitre plate, with further 2-fold dilutions across that row. The first well therefore contains 100 nL CSTx, the next well 50 nL, etc. If the first well shows lethality but the second well not, this implies a sensitivity of S = 71 nL/well (the LD50), which is the geometric midpoint between 100 and 50 nL/well. Correction by half a dilution step gives MLD = $2^{½}$ × S = 100 nL/well. If a given test toxin gives an MLD of 20 nL/well, this means a relative toxicity of 5. This method can be applied to each row individually or to the plate as a whole by maximum likelihood (ML) methods which optimise the parameters of interest for all rows simultaneously. In this report ML estimators are used because they have the advantage that rows with 100 % lethality or survival can be taken into account whereas this is not possible for individual rows. More details on the ML method used are given in Appendix 5.

If, in a similar way, the sensitivity of mice is determined it is possible to define a threshold where the number of dead wells translates to a prediction whether a mouse dies at that dose. For example, if the sensitivity of Vero cells is 71 nL/well and in mice 1 000 nL/mouse, then 3 dead wells would predict a dead mouse because the 3rd well contains about $2^{½}$ × 71 nL = 100 nL detecting toxin and therefore the 1st well contains 400 nL reference toxin. Since the total volume injected in mice is 0.5 mL instead of 0.1 mL in the wells, this implies 2 000 nL toxin per mouse which is above the mouse sensitivity and therefore predicted to be lethal. 2 dead wells would coincide with the mouse LD50 and 1 dead well would predict survival.

This method applied to the sensitivity tests of the 11 participating laboratories yields results as listed in Table 1. Shown, for both methods, are the MLD, the sensitivity expressed as LD50 in nL of the detecting toxin (CSTx) per experimental unit (mice or wells) and the ratio of these quantities (*in vivo/in vitro*).

Table 1 reveals large differences in sensitivity of the experimental units used by different laboratories. Laboratory 3 used the most sensitive mice and laboratory 5 the least sensitive, with a factor 12 difference in sensitivity which is more than 2 3-fold dilution steps. Laboratory 3 used the most sensitive Vero cells and laboratory 11 the least sensitive, with a factor 24 difference in sensitivity which is more than 4 2-fold dilution steps. The predictive ratios vary from 760 in laboratory 3 to 2 930 in laboratory 5.

Table 1 – *Sensitivity expressed as LD50 (in nL of CSTx per experimental unit) and as MLD (in dilution of CSTx per experimental unit)*

Lab	*In vivo*		*In vitro*		Ratio (*in vivo/ in vitro*)
	LD50 (nL CSTx/mouse)	MLD (CSTx dilution)	LD50 (nL CSTx/well)	MLD (CSTx dilution)	
1	237	1 215	–	–	–
2	288	1 000	0.198	357 000	1450
3	96	3 000	0.126	562 000	760
4	356	810	0.405	175 000	880
5	1 188	243	0.405	174 000	2930
6	617	468	0.757	93 000	820
7	–	–	0.196	361 000	–
8	–	–	0.236	300 000	–
9	–	–	1.694	42 000	–
10	–	–	0.134	529 000	–
11	–	–	3.049	23 000	–

Step 2: latent toxicity testing of test materials

Laboratories 1 to 6 performed the residual toxicity test in mice of the antitoxin (VI) and all toxoids. All laboratories used 5 IU/mL of VI and a 1/10 dilution of the toxoids. All mice in all tests survived, as expected.

Laboratories 2 to 11 performed the residual toxicity test on Vero cells of the VI and all toxoids except TdJ. All laboratories used 5 IU/mL of VI and a 1/10 dilution of the toxoids. The valid endpoints, expressed as average number of dead wells on a row, are summarised in Table 2. Also shown is the average endpoint per laboratory as a measure of sensitivity, and per toxoid as a measure of residual toxicity. The table shows that VI exhibits no latent toxicity in any laboratory. TdG shows most latent toxicity in all laboratories except in laboratories 4 and 9 where it is TdM showing most latent toxicity. The table also shows that laboratory 6 has by far the most sensitive Vero cells, contrary to what would be expected on the basis of Table 1. This apparent contradiction may be explained by the assumption that latent toxicity most likely includes non-specific toxic effects of the matrix and that a 1/10 dilution is not sufficiently high to 'dilute out' these matrix effects. In contrast, a dilution factor in the range of 20 000 to 500 000 in the MLD test is high enough to eliminate all non-specific toxic effects of the matrix. Therefore, it could be conclude that the Vero cell line used by laboratory 6 is the most sensitive cell line to the non-specific toxicity of the matrix while it is not the most sensitive to *C. septicum* toxin (see also section 6).

Before the study started it was expected that no toxoid should induce more than 3 dead wells but the results from laboratory 6 show at least that number for any toxoid and up to 6 dead wells for TdG. This, and the inconsistency with sensitivity tests by the same laboratory, is a potential problem for the validation of this method. The laboratory was contacted to ensure that the toxoids were prediluted 1/10, which they confirmed. However, it was noted that the preliminary 5-fold Vero cell sensitivity assay was much more sensitive (MLD = 256 000) than the 3-fold tests on which Table 1 is based (MLD = 93 000). This could mean that something went wrong with the 3-fold tests or that the CSTx had lost toxicity. The laboratory reported that they had indeed observed a shift over time in their full MLD and TCP tests and gave as possible explanation a limited stability of the toxin at + 4 °C. Other laboratories also reported concerns about the stability of the CSTx. Unfortunately there is no way to correct for drift with the chosen assay design so all calculations in this report are based on an assumed stable toxicity and sensitivity. Further studies may be required to investigate the importance to control and correct by design for drift of these parameters.

Step 3: preliminary ranging of test materials

This part of the study was mostly intended to perform preliminary tests to determine the optimal dose range of the toxins and toxoids to be used in step 4 of the study. No detailed discussion of these results will be presented here. This step was also intended to determine whether the sug-

Table 2 – *Summary of valid endpoints of the residual toxicity tests on Vero cells, expressed as average number of dead wells on a row*

Laboratory	VI	TdG	TdH	TdK	TdL	TdM	Average
2	0	3	2	0	0	2	1.2
3	0	3	1½	0	1½	2	1.3
4	0	1	½	1	0	2	0.8
5	0	2½	¼	½	0	2½	0.9
6	0	5¾	4	3¾	4¾	3¾	3.7
7	0	3	1	1	0	2	1.2
8	0	4	2	2	2½	2	2.1
9	0	1	1	1	1	3	1.2
10	0	3⅔	2	⅓	2⅙	2	2.0
11	0	4	2	3	3	1	2.2
Overall average	**0**	**3.1**	**1.6**	**1.3**	**1.5**	**2.2**	**1.6**

gested L^+ value of 1/170 was suitable for the mice used by laboratories 1 to 6. This turned out to be the case for laboratories 1 to 4, but not for laboratories 5 and 6 which established a value of 1/133.3 and 1/143 respectively. As a consequence they used these dilutions for the TCP assays in mice and on Vero cells whereas all other laboratories used the default value of 1/170.

Step 4: full testing of test materials

This step constitutes the main part of the study. It covers all MLD and TCP tests in mice and Vero cells. A complete overview of data from valid assays is provided in Appendix 7 (Tables A (MLD in mice), B (MLD on Vero cells), C (TCP in mice), D (TCP on Vero cells) and E (VI test on Vero cells)).

Table 3 – *Estimated MLD values (dilution factor) obtained in the mouse assay*

Lab	Test	TxA	TxB	TxC	TxD	TxE	TxF	CSTx
1	1	81	50	9.0	9.0	150	150	–
	2	81	50	3.0	9.0	150	150	–
	3	81	50	3.0	9.0	150	150	–
	GM	81	50	4	9	150	150	1215
	GCV	0	0	70	0	0	0	–
2	1	50	150	9.0	30	150	150	–
	2	150	150	5.2	17	150	87	–
	3	150	150	9.0	30	150	150	–
	GM	104	150	7	25	150	125	815
	GCV	70	0	33	33	0	33	–
3	1	450	300	14	24	300	300	–
	2	300	300	18	30	520	300	–
	3	173	200	14	30	200	200	–
	GM	286	262	15	28	315	262	3000
	GCV	51	24	15	13	51	24	–
4	1	36	12	3.0	5.2	12	12	–
	2	36	36	3.0	9.0	12	12	–
	3	36	36	n.p.	3.0	36	12	–
	GM	36	25	3	5	17	12	810
	GCV	0	70	0	59	70	0	–
5	1	10	45	3.0	9.0	135	78	–
	2	30	15	1.0	3.0	26	45	–
	3	30	45	n.p.	9.0	135	135	–
	GM	21	31	2	6	78	78	243
	GCV	70	70	91	70	121	59	–
6	1	84	51	5.7	10	153	88	–
	2	84	153	9.9	18	153	153	–
	3	84	153	9.9	18	153	153	–
	GM	84	106	8	15	153	127	468
	GCV	0	70	33	33	0	33	–
Overall GM		**73**	**74**	**5**	**12**	**128**	**110**	**–**
Inter-lab GCV		**113**	**117**	**91**	**81**	**69**	**75**	**–**
Median intra-lab GCV		**25**	**47**	**33**	**33**	**25**	**28**	**–**

n.p. = not performed.

Figure 1 – *Scatter plot of MLD results (in vivo) per lab and per toxin. Absolute values without correction for CSTx*

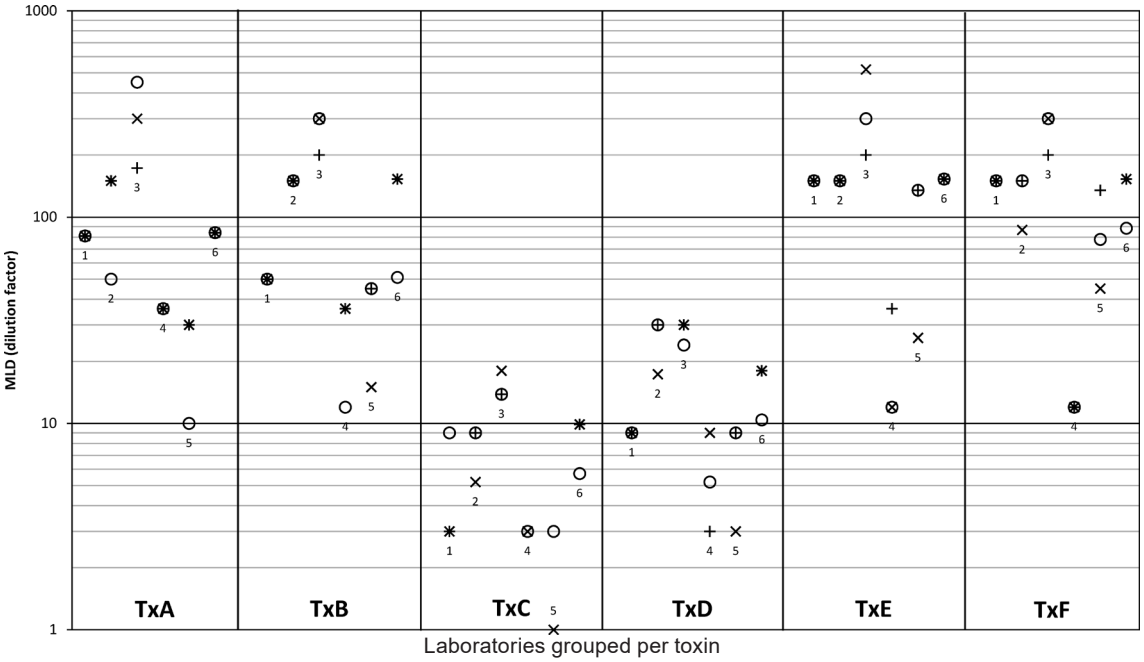
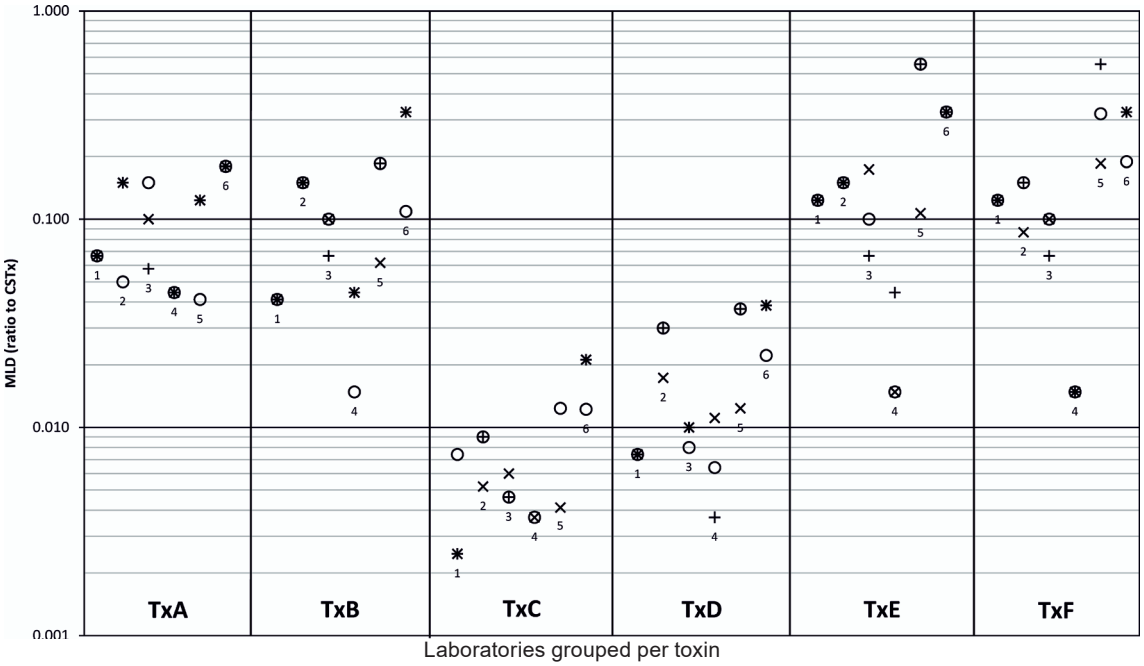


Figure 2 – *Scatter plot of MLD results (in vivo) per lab and per toxin. Relative toxicities expressed as MLD ratio with respect to CSTx*



MLD assay in mice

Laboratories 1 to 6 carried out their routine MLD method in mice. The 6 toxins were pre-diluted to an initial dilution determined in the preliminary ranging tests with further 3-fold dilution steps yielding 5 dose levels to be administered to 2 mice per dose level (0.5 mL per mouse). All laboratories carried out 3 valid assays per toxin, except laboratories 4 and 5 which carried out only 2 valid assays with TxC because there was not enough to perform an additional assay. Laboratory 3 performed a 4th valid assay with TxC and TxD because assay 1 was invalid on Vero cells. Although this extra assay was not strictly necessary and could be ignored in further

evaluations, it was decided to retain assays 2 to 4 and ignore assay 1 so as to keep them paired with the valid Vero cell assays. A summary overview of valid assays is given in Appendix 7, Table A. The table shows for each valid assay the pre-dilution factor used, the working dilutions and the responses (D = dead, L = alive).

The MLD in mice is defined as the highest dilution still causing the death of both mice. Applied to the data listed in Appendix 7, Table A this gives MLD values summarised in Table 3. Shown, for each assay, are the MLD value (reciprocal of dilution factor), the geometric mean (GM) of the valid assays and the geometric coefficient of variation (GCV). Also listed, for each laboratory, is the MLD of the CSTx as obtained in step 1 of the study. At the bottom of the table are given the overall GM (of the GM per laboratory), the overall GCV as a measure of reproducibility and the median GCV as a measure of repeatability.

The values in Table 3 are represented graphically in Figure 1 as absolute values without correction for sensitivity of the mouse used. The table and figure show that TxC and TxD are systematically identified to be of low toxicity and the dispersion of results is similar for all toxins,

Table 4 – *Estimated MLD values obtained in the mouse assay relative to CSTx*

Lab	Test	TxA	TxB	TxC	TxD	TxE	TxF
1	1	0.067	0.041	0.007	0.007	0.123	0.123
	2	0.067	0.041	0.002	0.007	0.123	0.123
	3	0.067	0.041	0.002	0.007	0.123	0.123
	GM	0.067	0.041	0.004	0.007	0.123	0.123
	GCV	0	0	70	0	0	0
2	1	0.050	0.150	0.009	0.030	0.150	0.150
	2	0.150	0.150	0.005	0.017	0.150	0.087
	3	0.150	0.150	0.009	0.030	0.150	0.150
	GM	0.104	0.150	0.007	0.025	0.150	0.125
	GCV	70	0	33	33	0	33
3	1	0.150	0.100	0.005	0.008	0.100	0.100
	2	0.100	0.100	0.006	0.010	0.173	0.100
	3	0.058	0.067	0.005	0.010	0.067	0.067
	GM	0.095	0.087	0.005	0.009	0.105	0.087
	GCV	51	24	15	13	51	24
4	1	0.044	0.015	0.004	0.006	0.044	0.044
	2	0.044	0.044	0.004	0.011	0.044	0.044
	3	0.044	0.044	n.p.	0.004	0.133	0.044
	GM	0.044	0.031	0.004	0.006	0.064	0.044
	GCV	0	70	0	59	70	0
5	1	0.041	0.185	0.012	0.037	0.556	0.321
	2	0.123	0.062	0.004	0.012	0.107	0.185
	3	0.123	0.185	n.p.	0.037	0.556	0.556
	GM	0.086	0.128	0.007	0.026	0.321	0.321
	GCV	70	70	91	70	121	59
6	1	0.179	0.109	0.012	0.022	0.327	0.189
	2	0.179	0.327	0.021	0.038	0.327	0.327
	3	0.179	0.327	0.021	0.038	0.327	0.327
	GM	0.179	0.227	0.018	0.032	0.327	0.272
	GCV	0	70	33	33	0	33
Overall GM		**0.088**	**0.089**	**0.006**	**0.014**	**0.153**	**0.132**
Inter-lab GCV		**49**	**91**	**65**	**82**	**72**	**84**
Median intra-lab GCV		**25**	**47**	**33**	**33**	**25**	**28**

n.p. = not performed.

with inter-lab GCVs ranging from 81 % to 146 % and median intra-laboratory GCVs ranging from 25 % to 47 %.

Since sensitivity of the mice affects the absolute MLD value, the values were also evaluated when corrected for the MLD of the CSTx. Table 4 shows the ratio of the MLD of the test toxins to the MLD of the CSTx. A graphical representation is given in Figure 2. A slight improvement in reproducibility can be detected for TxA, TxB and TxC but no improvement can be detected for TxD, TxE and TxF. Where the inter-laboratory GCVs range from 69 % to 117 % without correction for sensitivity, the inter-laboratory GCVs range from 49 % to 91 % when corrected for sensitivity. The intra-laboratory variation is, of course, not affected by this correction.

MLD assay on Vero cells

Laboratories 2 to 11 carried out the MLD assays on Vero cells. Each laboratory was requested to produce 3 valid assays for each test toxin. Invalid assays had to be repeated but the laboratories were requested to report results from invalid assays to assess the incidence of invalid assays.

Table 5 shows the testing schedule of the laboratories.

Table 5 – *Testing schedule of MLD assays on Vero cells per laboratory*

Laboratory	TxA	TxB	TxC	TxD	TxE	TxF
2	1;2;3	1;2;3	1;2;3	1;2;3	1;2;3	1;2;3
3	1;2;3	1;2;3	(1);2;3;4	(1);2;3;4	1;2;3	1;2;3
4	1;2;4	1;2;4	1;(2);3;[4];6	1;[2];5;6	2;4;5	3;4;5
5	(1);3;4;5	1;3;4	3;5;(6);[7];8	(3);5;5;6	2;4;5	2;(4);5;6
6	(1);[2];(3);4;5;6	1;2;3	4;5;6	4;5;6	1;2;3	1;2;3
7	1;2;3	1;2;3	1;2;3	1;2;3	1;2;3	1;2;3
8	(2?);3;4;5	1;2;4	1;2;4	1;3;4	2;3;4	2;3;4
9	1;2;2	1;2;2	1;2;2	1;2;2	1;2;2	1;2;2
10	1;2;3	1;2;3	1;2;3	1;2;3	1;2;3	1;2;3
11	1;3;5	(1);4;6;8	2;4;6	(2);4;6;7	1;(3);5;7	3;5;8

Numbers indicate the day of testing within a laboratory. Invalid tests are shown between brackets. All endpoints from these assays are listed in Appendix 7, Table B.

The table shows that laboratories 2, 7 and 10 performed all valid assays on 3 different days, each assay including all toxins. Laboratory 3 repeated one assay on TxC and TxD. Laboratory 4 repeated 1 invalid assay on TxC. Two other tests were valid according to the criteria specified in the protocol, but the laboratory had doubts about the quality of these assays and repeated them (shown between square brackets). Laboratory 5 repeated one invalid test for TxA, TxC, TxD and TxF. One test for TxC was valid according to the criteria specified in the protocol but was repeated because the endpoints seemed inconsistent with the other assays. Laboratory 6 tested toxins TxA, TxB, TxE and TxF together on 3 different days. 2 of these assays with TxA were invalid so this toxin was tested together with TxC and TxD on 3 other days. Laboratory 8 performed the assays over 5 different days. In order to reduce its delay in completing the testing, Laboratory 9 performed assays 2 and 3 for all toxins on the same day but using independent test sample dilution series. Laboratory 11 repeated one assay for TxB due to insufficient cell harvest for 2 plates, and 1 assay for TxD and TxE each because of a too high CV. Overall, the incidence of invalid assays is 17/197 or about 9 %.

A summary overview of valid assays is given in Appendix 7, Table B. The table shows for each valid assay the pre-dilution factor used, the working dilutions and the responses per row (1 to 9 = number of dead wells, D = all wells dead, L = all wells alive). Also shown on the left hand side of the table is the dilution factor of the CSTx on the control row. Using the ML method described in Appendix 5, each assay yields an estimate of the MLD of the test toxin and of the CSTx on the control row. These values, expressed as dilution factor, are listed in Table 6 together with the GM and GCV of the 3 tests per toxin. Also shown are the overall GM (of the GM per labora-

tory), the overall GCV as a measure of reproducibility and the median GCV as a measure of repeatability.

The values in Table 6 are represented graphically in Figure 3 as absolute values without correction for sensitivity of the Vero cells used. The table and figure show that TxC and TxD are systematically identified to be of low toxicity. This observation is consistent with the assay in mice. The dispersion of results is similar for all toxins with inter-laboratory GCVs ranging from 143 % to 183 % and median intra-laboratory GCVs ranging from 24 % to 50 %.

Table 6 – *Estimated MLD values (dilution factor) obtained in the Vero cell assay*

Lab	Test	TxA	CSTx	TxB	CSTx	TxC	CSTx	TxD	CSTx	TxE	CSTx	TxF	CSTx	CSTx Overall GM
2	1	22186	320000	46139	320000	1434	226274	6561	320000	54986	226274	54986	320000	–
	2	19184	452548	35678	320000	655	226274	3326	320000	25798	160000	22186	226274	–
	3	54986	320000	30222	226274	1674	320000	2184	226274	38844	320000	35678	226274	–
	GM	28604	359188	36779	285088	1163	253984	3626	285088	38053	226274	35176	253984	274318
	GCV	62	20	22	20	54	20	60	20	39	36	48	20	–
3	1	35678	226274	42412	320000	3069	640000	5708	640000	30990	320000	38362	320000	–
	2	60442	640000	38362	320000	1996	452548	12035	640000	100672	640000	77693	640000	–
	3	30990	320000	37526	320000	2479	452548	3326	320000	53856	640000	66525	640000	–
	GM	40581	359188	39377	320000	2476	507968	6114	507968	55181	507968	58312	507968	443918
	GCV	36	57	7	0	22	20	72	42	64	42	38	42	–
4	1	7482	192000	5285	135765	595	192000	2149	192000	26392	135765	28399	96000	–
	2	8020	192000	3990	135765	251	96000	3260	192000	35704	192000	33021	192000	–
	3	9880	384000	12167	271529	595	192000	2470	192000	33021	192000	30598	192000	–
	GM	8401	241905	6354	171053	446	152391	2587	192000	31453	171053	30615	152391	177768
	GCV	15	42	63	42	53	42	21	0	16	20	8	42	–
5	1	8129	194400	4803	194400	251	194400	3884	388800	17544	97200	23166	194400	–
	2	7568	388800	5626	194400	251	388800	3884	388800	17544	388800	46566	388800	–
	3	7040	388800	6072	388800	509	388800	5034	388800	20160	388800	37698	388800	–
	GM	7566	308591	5475	244929	318	308591	4235	388800	18376	244929	34388	308591	296933
	GCV	7	42	12	42	42	42	15	0	8	95	37	42	–
6	1	52455	320000	17043	80000	1767	226274	7650	226274	60352	80000	52171	80000	–
	2	13964	320000	10473	113137	532	320000	7100	320000	25856	80000	36620	113137	–
	3	13964	160000	10473	160000	377	226274	4959	160000	20993	113137	39282	113137	–
	GM	21707	253984	12319	113137	708	253984	6458	226274	31997	89797	42181	100794	156949
	GCV	89	42	29	36	96	20	23	36	61	20	19	20	–

Lab	Test	TxA	CSTx	TxB	CSTx	TxC	CSTx	TxD	CSTx	TxE	CSTx	TxF	CSTx	CSTx Overall GM
7	1	30527	200000	40705	200000	2544	200000	5453	200000	50129	282843	43201	200000	–
	2	35072	282843	46762	400000	1925	400000	6716	400000	40705	400000	61132	400000	–
	3	23102	400000	33032	400000	1452	282843	3589	400000	35420	400000	37597	400000	–
	GM	29136	282843	39764	317480	1923	282843	5085	317480	41654	356359	46306	317480	311426
	GCV	22	36	18	42	29	36	33	42	18	20	25	42	–
8	1	31328	540000	19252	381838	1880	540000	2160	381838	41298	381838	54533	540000	–
	2	47528	540000	17948	540000	651	540000	1636	540000	58456	540000	54533	381838	–
	3	25442	270000	16728	540000	1754	270000	5356	540000	67164	540000	88681	540000	–
	GM	33585	428598	17946	481085	1290	428598	2665	481085	54529	481085	64128	481085	462912
	GCV	33	42	7	20	65	42	68	20	25	20	29	20	–
9	1	2871	20000	2871	20000	69	20000	86	20000	3017	20000	1487	20000	–
	2	2871	28284	2162	28284	69	20000	223	28284	3017	20000	2011	20000	–
	3	1372	20000	1372	20000	69	20000	206	20000	2058	20000	1372	20000	–
	GM	2245	22449	2042	22449	69	20000	158	22449	2656	20000	1601	20000	21189
	GCV	45	20	39	20	0	0	57	20	22	0	20	0	–
10	1	67164	960000	95063	960000	4137	960000	8273	960000	88681	960000	50881	1357645	–
	2	62656	960000	54533	480000	2544	480000	7200	480000	67164	480000	47467	480000	–
	3	50881	678823	41298	678823	1673	480000	5846	480000	62656	678823	44277	480000	–
	GM	59825	855263	59822	678823	2601	604762	7035	604762	71996	678823	47465	678823	678823
	GCV	15	20	44	36	48	42	18	42	19	36	7	66	–
11	1	6198	362039	4437	256000	268	256000	710	64000	11158	362039	7142	256000	–
	2	5022	128000	2342	64000	98	64000	1004	64000	2686	64000	4369	90510	–
	3	3802	64000	3099	128000	173	90510	874	64000	7672	64000	6653	64000	–
	GM	4909	143675	3182	128000	166	114035	854	64000	6127	114035	5921	114035	109727
	GCV	25	107	33	79	54	83	18	0	85	131	27	83	–
Overall GM		**15901**		**13292**		**680**		**2694**		**25201**		**25617**		**–**
Inter-lab GCV		**145**		**176**		**183**		**173**		**143**		**174**		**–**
Median Intra-lab GCV		**29**		**25**		**50**		**28**		**24**		**26**		**–**

Since sensitivity of the Vero cells affects the absolute MLD value, the values were also evaluated when corrected for the MLD of the CSTx. For this purpose the average MLD of the CSTx across all plates was used. Table 7 shows the ratio of the MLD of the test toxins to the average MLD of the CSTx. A graphical representation is given in Figure 4. A clear improvement of reproducibility can be detected for all toxins. Where the inter-laboratory GCVs range from 143 % to 183 % without correction for sensitivity, the inter-laboratory GCVs range from 43 % to 77 % when corrected for sensitivity.

Figure 3 – *Scatter plot of MLD results (in vitro) per lab and per toxin. Absolute values without correction for sensitivity*

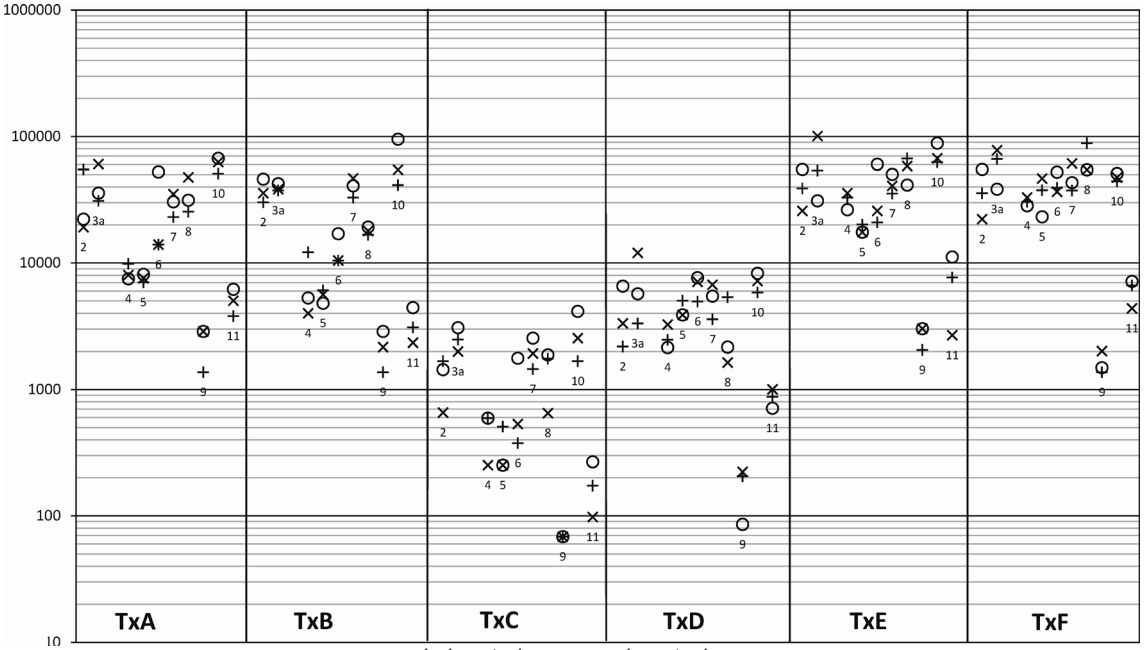
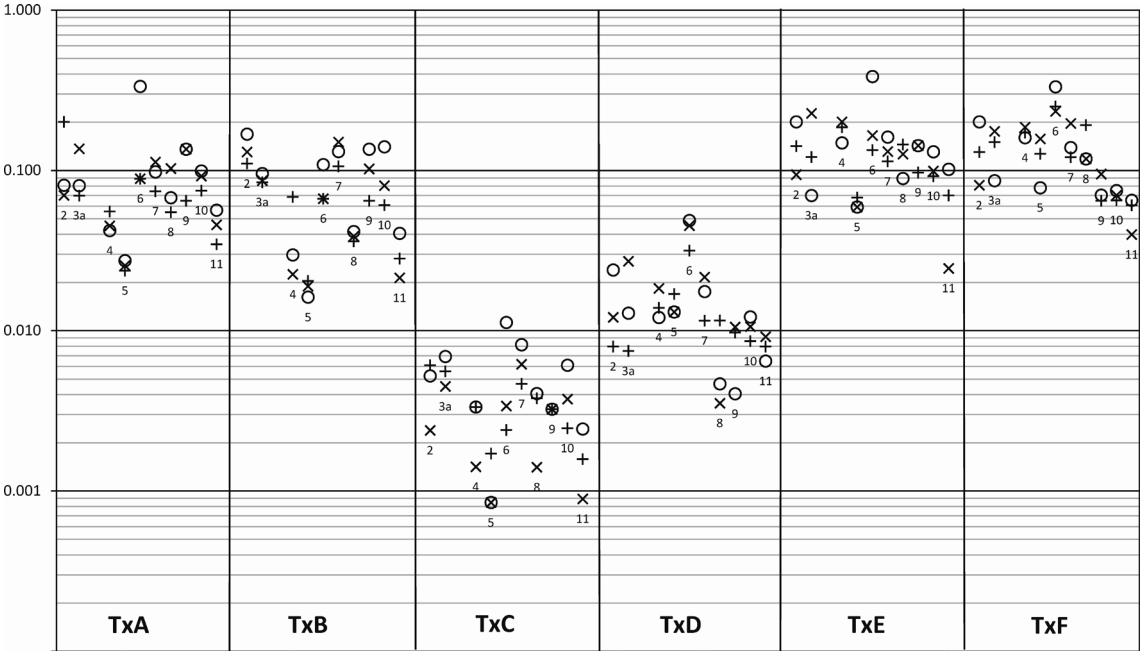


Table 7 – *Estimated MLD values of test toxins expressed as ratio to the average MLD per lab of the CSTx obtained in the Vero cell assay*

Lab	Test	TxA	TxB	TxC	TxD	TxE	TxF
2	1	0.081	0.168	0.005	0.024	0.200	0.200
	2	0.070	0.130	0.002	0.012	0.094	0.081
	3	0.200	0.110	0.006	0.008	0.142	0.130
	GM	0.104	0.134	0.004	0.013	0.139	0.128
	GCV	62	22	54	60	39	48
3	1	0.080	0.096	0.007	0.013	0.070	0.086
	2	0.136	0.086	0.004	0.027	0.227	0.175
	3	0.070	0.085	0.006	0.007	0.121	0.150
	GM	0.091	0.089	0.006	0.014	0.124	0.131
	GCV	36	7	22	72	64	38
4	1	0.042	0.030	0.003	0.012	0.148	0.160
	2	0.045	0.022	0.001	0.018	0.201	0.186
	3	0.056	0.068	0.003	0.014	0.186	0.172
	GM	0.047	0.036	0.003	0.015	0.177	0.172
	GCV	15	63	53	21	16	8
5	1	0.027	0.016	0.001	0.013	0.059	0.078
	2	0.025	0.019	0.001	0.013	0.059	0.157
	3	0.024	0.020	0.002	0.017	0.068	0.127
	GM	0.025	0.018	0.001	0.014	0.062	0.116
	GCV	7	12	42	15	8	37
6	1	0.334	0.109	0.011	0.049	0.385	0.332
	2	0.089	0.067	0.003	0.045	0.165	0.233
	3	0.089	0.067	0.002	0.032	0.134	0.250
	GM	0.138	0.078	0.005	0.041	0.204	0.269
	GCV	89	29	96	23	61	19
7	1	0.098	0.131	0.008	0.018	0.161	0.139
	2	0.113	0.150	0.006	0.022	0.131	0.196
	3	0.074	0.106	0.005	0.012	0.114	0.121
	GM	0.094	0.128	0.006	0.016	0.134	0.149
	GCV	22	18	29	33	18	25
8	1	0.068	0.042	0.004	0.005	0.089	0.118
	2	0.103	0.039	0.001	0.004	0.126	0.118
	3	0.055	0.036	0.004	0.012	0.145	0.192
	GM	0.073	0.039	0.003	0.006	0.118	0.139
	GCV	33	7	65	68	25	29
9	1	0.136	0.136	0.003	0.004	0.142	0.070
	2	0.136	0.102	0.003	0.011	0.142	0.095
	3	0.065	0.065	0.003	0.010	0.097	0.065
	GM	0.106	0.096	0.003	0.007	0.125	0.076
	GCV	45	39	0	57	22	20
10	1	0.099	0.140	0.006	0.012	0.131	0.075
	2	0.092	0.080	0.004	0.011	0.099	0.070
	3	0.075	0.061	0.002	0.009	0.092	0.065
	GM	0.088	0.088	0.004	0.010	0.106	0.070
	GCV	15	44	48	18	19	7
11	1	0.056	0.040	0.002	0.006	0.102	0.065
	2	0.046	0.021	0.001	0.009	0.024	0.040
	3	0.035	0.028	0.002	0.008	0.070	0.061
	GM	0.045	0.029	0.002	0.008	0.056	0.054
	GCV	25	33	54	18	85	27
Overall GM		**0.073**	**0.061**	**0.003**	**0.012**	**0.116**	**0.118**
Inter-lab GCV		**55**	**77**	**60**	**59**	**43**	**50**
Median Intra-lab GCV		**29**	**25**	**50**	**28**	**24**	**26**

n.p. = not performed.

Figure 4 – *Scatter plot of MLD results (in vitro) per lab and per toxin. Relative toxicities expressed as MLD ratio with respect to CSTx*



Another approach is to establish for laboratory 2 to 6 a cut-off which translates the endpoint on each individual row to a prediction of the response in mice. These predictions would then be used as if obtained from a genuine mouse assay to establish the MLD in the usual way. For this approach it is essential to establish as accurately as possible the cut-off for each laboratory. One way to do this is to use the ratio of the MLD *in vivo* to the MLD *in vitro* of the detecting toxin (CSTx) but due to the limited amount of data for the CSTx this approach cannot be expected to be very accurate. Instead it was decided to establish a consensus threshold based on the pooled set of toxins within laboratories. This approach can be justified because a useful cut-off should not depend on the toxin under investigation. On the other hand, from the purely statistical point of view it is not desirable to use the data itself to establish a parameter which is then plugged back into the model to analyse the very same dataset. Although the effect of this auto-dependency is probably not very big it should be kept in mind that the outcome has to be regarded as a best case scenario.

The probit model was used to determine the cut-off per laboratory that gives the best prediction of the responses in mice. The optimal cut-offs established this way are 189, 141, 475, 264 and 172 for laboratories 2 to 6 respectively. For example: laboratory 2 found in assay 1 for TxA 8 dead wells on the second row (see Appendix 7, Table B). The LD50 on this row is therefore $50 \times 3 \times 2^{7.5}/189 = 144$. This row corresponds with mice that received a 1/150 dose of the toxin which is slightly weaker than the cut-off of 1/144 and therefore predicts survival. Appendix 7, Table F, shows a complete overview of the predicted responses in mice based on the observed responses on Vero cells. The resulting MLDs are listed in Table 8. The table shows that TxC and TxD are still identified as the least toxic samples, but the ranking is not exactly the same as in the real *in vivo* estimates. Repeatability (intra-laboratory GCV) and reproducibility are not systematically improved when compared to Table 3.

Table 8 – *Predicted MLD values in vivo based on in vitro testing*

Lab	Test	TxA	TxB	TxC	TxD	TxE	TxF
2	1	50	150	3.0	30	150	150
	2	50	150	3.0	10	87	50
	3	150	150	3.0	< 10	150	150
	GM	72	150	3	17	125	104
	GCV	70	0	0	91	32	70
3	1	150	300	24	42	300	300
	2	300	300	18	90	900	520
	3	300	200	24	30	346	600
	GM	238	262	22	48	454	454
	GCV	42	24	17	61	65	38
4	1	12	12	1.0	3.0	36	36
	2	12	4	< 1.0	9.0	36	36
	3	12	12	1.0	3.0	36	36
	GM	12	8	1	4	36	36
	GCV	0	70	0	70	0	0
5	1	30	15	< 1.0	9.0	45	135
	2	30	15	< 1.0	16	45	135
	3	90	15	1.0	27	135	135
	GM	43	15	1	16	65	135
	GCV	70	0	n.a.	59	70	0
6	1	252	88	9.9	54	459	459
	2	84	51	4.3	54	153	153
	3	84	51	3.3	31	153	265
	GM	121	61	5	45	221	265
	GCV	70	32	62	33	70	59
Overall GM		**64**	**50**	**3**	**19**	**124**	**143**
Inter-lab GCV		**161**	**278**	**206**	**127**	**130**	**124**
Median intra-lab GCV		**70**	**24**	**8**	**61**	**65**	**38**

n.a. = not applicable.

Toxin/antitoxin (VI) test

The toxin/antitoxin test on Vero cells has to be carried out in parallel with the TCP tests on Vero cells. A complete listing of endpoints is provided in Appendix 7, Table E.

The toxin/antitoxin test aims at quantifying the toxin equivalence of the detecting toxin (CSTx) in combination with the sensitivity of the Vero cells. Ideally the sensitivity of the Vero-cells should depend only on the level of remaining toxin and not on the presence of the antitoxin, bound or unbound. If this assumption is fulfilled, the observed sensitivity in the MLD assays can be used to calculate the toxin equivalence of the toxin/antitoxin combination.

The following example illustrates this. 1 mL of 1/170 diluted CSTx is added to 1 mL antitoxin at 1.5 IU/mL. This 2 mL mix is further diluted 1/16 and 0.1 mL is loaded into the first well of the first row of the plate, with further 2-fold dilutions across that row. Let us assume that the observed sensitivity in the MLD test was S = 0.35 nL/well and that the first 3 wells of the row show lethality. The 3rd well is therefore estimated to contain $0.35 \times 2^{½} = 0.50$ nL toxin and the 1st well about 2 nL. The original tube must therefore have contained $2 \times 20 \times 16 = 640$ nL toxin. The original amount of toxin added was 1 mL/170 = 5882 nL so 5242 nL must have been neutralised by the 1.5 IU antitoxin. This yields a toxin equivalence (N) of 5242/1.5 = 3495 nL/IU or equivalently N = 286 IU/mL. This method can be applied to each row individually or to the plate as a whole by ML methods which optimise the parameters of interest for all rows simultaneously. In this report ML estimators are used because they have the advantage that rows with 100 % lethality

or survival can be taken into account whereas this is not possible for individual rows. More details on the ML method used are given in Appendix 5.

Interestingly, laboratories 6 and 10 included an extra row on all TCP plates with 2 IU antitoxin, 1 mL of L+ diluted toxin and without toxoid. In laboratory 6 this resulted in 0 to 5 dead wells after 1/16 pre-dilution of the mix. This high variability can be explained if very low quantities of toxin remain and almost all the toxin is neutralised by the antitoxin. Since the L+ used in this laboratory is 1/143 it would mean that 1 mL/143 = 6 993 nL is almost completely neutralised by 2 IU antitoxin. This gives a toxin equivalence of slightly more than 3 496 nL/IU or, equivalently, slightly less than 286 IU/mL. In laboratory 10 an L+ of 1/170 was used giving between 0 and 2 dead wells after 1/16 pre-dilution of the mix. If the toxin equivalence is indeed more than 3 496 nL/IU, all toxin should be neutralised by the 2 IU antitoxin since there was only 1 mL/170 = 5 882 nL toxin present initially. This is a contradiction and would indicate that other components play a role in the lethal effect of the toxin/antitoxin mix. On the other hand, the fact that assays 2 and 3 from laboratory 10 show no lethality at all after 1/8 pre-dilution, might point to an anomaly with assay 1 only and that complete neutralisation was indeed achieved.

Table 9 shows the results of the simultaneous optimisation of the toxin equivalence of the CSTx and the sensitivity of the Vero cells. It also shows the estimated sensitivity of the Vero cells conditional on an assumed toxin equivalence of 284 IU/mL. It should be mentioned here that the calculated sensitivity per laboratory does not depend very much on the assumed value of the toxin equivalence. In general there is less than 10 % difference in calculated sensitivity when a toxin equivalence of 3600 nL/IU instead of 3500 nL/IU is assumed. Since 10 % is less than the 2-fold steps used in the assay, the impact of the exact choice of this value will not be of practical importance for the global outcome of the study. All further calculations will be based on the average toxin equivalence of N = 284 IU/mL. It may be useful to confirm this assumption in an assay specifically designed to quantify this value with more precision.

Surprisingly, the inter-laboratory variation of the toxin equivalence values is, with a GCV of 7 %, much lower than that of the MLD values which are in the range of 43 % to 77 % (see Table 7). The current study was not set up to express the toxicity of the test toxins in terms of toxin equivalence (i.e. expressed in IU/mL) instead of MLD, but there is no reason why the same principle would not be applicable to other toxins in addition to CSTx. This way of expressing toxicity could possibly further improve reproducibility of the method. A possible assay design optimised for this purpose is discussed in Appendix 6.

Table 9 – *Estimates of toxin equivalence (N) of CSTx and sensitivity (S) of the Vero cell lines based on the VI tests*

	Simultaneous optimisation		Fixed N = 284 IU/mL
Lab	**N (IU/mL)**	**S (nL/well)**	**S (nL/well)**
2	295	0.348	0.314
3a	291	0.154	0.144
3b	307	0.384	0.320
4	282	0.520	0.532
5	274	0.174	0.181
6	258	0.258	0.302
7	278	0.492	0.526
8	313	0.340	0.267
9	279	0.750	0.797
10	296	0.497	0.447
11	246	1.506	2.402
Average	**284**	**–**	**–**

Laboratory 3a shows the results obtained before application of isopropanol; laboratory 3b those obtained after application of isopropanol.

TCP assay in mice

Laboratories 1 to 6 carried out the TCP method in mice. Laboratories 2 to 6 used their routine method, but laboratory 1 reported that this type of test is not performed routinely in their laboratory and they were not able to produce coherent results. A summary overview of assays is given in Appendix 7, Table C. The table shows for each assay the TCP units per dose level and the responses (D = dead, L = alive). Also shown is for each laboratory the L^+ value. It can indeed be seen that the results from laboratory 1 are extremely incoherent. This, and the fact that the laboratory does not routinely perform the assay, was reason to exclude the TCP results from this laboratory from further evaluations.

The TCP value is defined as the dilution that causes the death of 1 mouse but not of the other, or as the midpoint between the dilution that causes the death of both mice and the adjacent dilution where both mice survive. The TCP values resulting from this definition are shown on top of each assay in Appendix 7, Table C, and are reprised in Table 10, together with the GM and the GCV of the valid assays. At the bottom of the table are given the overall GM (of the GM per laboratory), the overall GCV as a measure of reproducibility and the median GCV as a measure

Table 10 – *Estimated TCP values (IU/mL) obtained in the mouse assay*

Lab	Test	TdG	TdH	TdJ	TdK	TdL	TdM
1	1	75	30	10	70	< 25	< 25
	2	< 60	10	10	> 115	> 75	> 75
	3	> 130	65	10	> 215	155	> 215
	GM	n.c.	27	10	n.c.	n.c.	n.c.
	GCV	n.a.	119	0	n.a.	n.a.	n.a.
2	1	110	50	20	140	60	40
	2	90	30	20	120	20	20
	3	70	30	< 10	90	10	20
	GM	88	36	20	115	23	25
	GCV	23	30	0	23	112	42
3	1	150	50	30	> 140	> 80	INV
	2	180	60	50	> 200	90	INV
	3	200	50	50	> 180	130	INV
	GM	175	53	42	n.c.	108	n.c.
	GCV	15	11	30	n.a.	26	n.a.
4	1	80	40	20	200	70	70
	2	130	40	20	190	80	40
	3	150	60	20	170	60	90
	GM	116	46	20	186	70	63
	GCV	34	24	0	8	14	43
5	1	170	80	30	250	100	90
	2	180	80	40	210	70	60
	3	200	60	20	200	70	70
	GM	183	73	29	219	79	72
	GCV	8	17	36	12	21	21
6	1	170	80	20	210	120	220
	2	180	80	20	210	120	220
	3	170	70	20	220	110	220
	GM	173	77	20	213	117	220
	GCV	3	8	0	3	5	0
Overall GM		**142**	**48**	**21**	**178**	**69**	**71**
Inter-lab GCV		**33**	**42**	**51**	**31**	**73**	**110**
Median intra-lab GCV		**15**	**20**	**0**	**10**	**21**	**31**

n.c. = not calculated. n.a. = not applicable. INV = invalid.

of repeatability. The inter-laboratory GCVs range from 31 % to 110 % and the median intra-laboratory GCVs range from 0 % to 31 %.

The values in Table 10 are represented graphically in Figure 5. The table and figure show that TdJ is generally, but not always, identified to be of lowest total combing power and that TdK is generally, but again not always, identified to be of highest total combining power. The dispersion of results is similar for all toxoids.

TCP assay on Vero cells

Laboratories 2 to 11 carried out the TCP assays on Vero cells. A summary overview of valid assays is given in Appendix 7, Table D. Each laboratory was requested to produce 3 valid assays for each toxoid. Invalid assays had to be repeated but the laboratories were requested to report results from invalid assays to assess the incidence of invalid assays. The testing schedule of the laboratories, as reported, is summarised in Table 11.

Figure 5 – *Scatter plot of TCP results (in vivo) per laboratory and per toxoid*

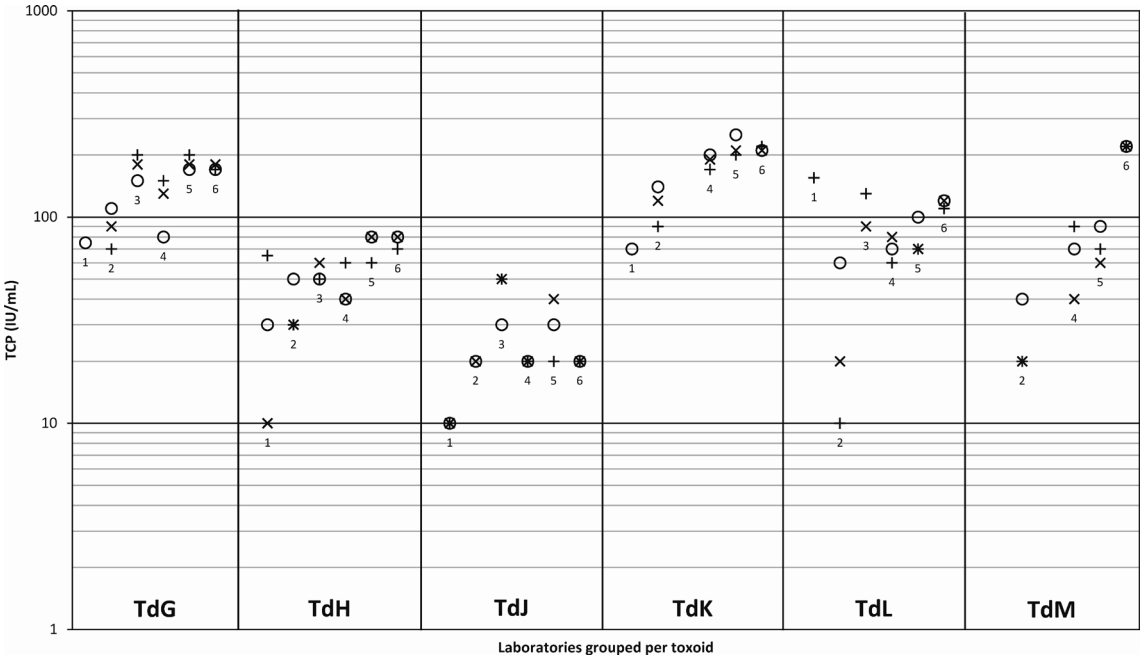


Table 11 – *Testing schedule of TCP assays on Vero cells per laboratory*

Lab	TdG	TdH	TdJ	TdK	TdL	TdM	VI
2	1;2;3	1;2;3	1;2;3	1;2;3	1;2;3	1;2;3	1;2;3
3	1;2;3	2;3;4	3;5;5	4;5;6	2;3;6	2;3;6	1;5;6 (+ prel.)
4	1;3;5	2;4;6	2;4;6	1;3;5	2;4;6	1;3;5	1;2;3;4;5;6
5	(1);2;3;4	1;2;3	1;2;3	2;(3);4;5	4;(5);6;7	(4);5;6;7	1;2;3;4;5;6;7
6	1;2;3	1;2;3	(1);(2);3;7;8	4;5;6	4;5;6	4;5;6	1;2;3;4;5;6;7;8
7	1;2;3	1;2;3	1;2;3	1;2;3	1;2;3	1;2;3	1;2;3
8	1;2;3	1;2;3	1;2;3	1;2;3	1;2;3	1;2;3	1;2;3
9	1;2;3;4	1;2;(3);4	1;2;3;4	1;2;3;4	1;2;3;4	1;2;3;4	1;2;3;4
10	1;2;3	1;2;3	1;2;3	1;2;3	1;2;3	1;2;3	1;2;3
11	(3);5;6;7	1;5;6	1;5;6	3;5;6	2;4;5	2;4;6	1;2;3;4;5;6;7

Numbers indicate the day of testing within a laboratory. Invalid tests are shown between brackets. All endpoints from these assays are listed in Appendix 7, Table D and Table E.

Table 12 – *Estimated TCP values (IU/mL) without prior information on sensitivity of Vero cells*

		TdG		TdH		TdJ		TdK		TdL		TdM	
Lab	Test	B	S	B	S	B	S	B	S	B	S	B	S
2	1	n.c.	0.000	41	0.157	40	0.674	53	0.000	30	0.001	39	0.071
	2	40	0.001	46	0.199	37	0.642	62	0.045	46	0.129	53	0.314
	3	80	0.380	79	0.805	48	1.985	190	0.790	73	0.558	65	0.982
	GM	56	0.002	53	0.293	41	0.951	86	0.026	46	0.045	51	0.280
	GCV	53	INF	36	109	13	71	79	99938	47	16663	26	216
3a	1	96	0.087	38	0.165	35	0.176	140	0.212	73	0.200	97	0.257
	2	212	0.204	43	0.116	39	0.111	138	0.099	98	0.400	n.c.	0.576
	3	200	0.237	69	0.213	45	0.310	218	0.136	135	0.409	136	0.287
	GM	160	0.162	48	0.160	39	0.182	161	0.142	99	0.320	115	0.349
	GCV	46	58	32	31	13	55	26	39	32	42	24	46
3b	1	94	0.361	40	0.830	32	0.166	182	0.289	73	0.200	60	0.255
	2	72	0.051	58	0.201	45	0.150	142	0.103	87	0.415	n.c.	0.576
	3	157	0.183	56	0.211	33	0.228	200	0.886	66	0.372	72	1.140
	GM	102	0.150	51	0.328	37	0.178	173	0.298	75	0.314	65	0.551
	GCV	41	130	20	95	19	22	18	148	14	41	13	87
4	1	180	0.806	49	0.720	32	0.844	135	0.426	90	0.854	102	1.980
	2	191	0.799	67	0.493	43	0.818	191	0.635	120	0.839	109	1.624
	3	170	0.407	61	0.833	43	0.818	131	0.306	97	0.816	108	0.996
	GM	180	0.640	58	0.666	39	0.827	150	0.436	102	0.836	106	1.474
	GCV	6	41	16	28	16	2	21	38	15	2	3	37
5	1	94	0.260	n.c.	0.365	123	0.819	123	0.260	62	0.257	121	0.365
	2	62	0.257	34	0.257	7	0.117	164	0.260	94	0.260	76	0.254
	3	110	0.259	7	0.117	26	0.504	123	0.260	62	0.257	86	0.258
	GM	86	0.259	16	0.222	29	0.364	135	0.260	71	0.258	93	0.288
	GCV	30	1	147	63	251	134	17	0	24	1	24	21
6	1	7	0.003	56	0.209	24	0.764	5	0.005	120	0.489	280	1.150
	2	253	0.483	40	0.242	33	0.429	n.c.	0.966	3	0.009	n.c.	1.932
	3	6	0.010	40	0.341	33	0.429	300	0.359	n.c.	0.298	13	0.018
	GM	22	0.025	45	0.259	29	0.520	40	0.121	19	0.110	61	0.345
	GCV	935	3179	20	25	20	34	5517	4863	2758	1034	996	2581
7	1	141	0.426	50	0.490	26	1.286	253	0.801	119	0.811	96	0.841
	2	145	0.412	49	0.421	26	1.286	134	0.298	134	0.809	96	0.841
	3	141	0.426	50	0.490	26	1.286	110	0.226	104	0.680	85	0.709
	GM	142	0.422	50	0.466	26	1.286	155	0.378	118	0.764	92	0.795
	GCV	2	2	0	9	0	0	46	75	13	10	7	10
8	1	190	0.287	89	0.242	111	0.581	n.c.	0.406	n.c.	0.406	n.c.	0.812
	2	190	0.287	90	0.347	n.c.	0.914	n.c.	0.812	n.c.	0.812	263	0.812
	3	n.c.	0.264	155	0.406	100	1.148	297	0.574	149	0.644	200	0.950
	GM	190	0.279	107	0.324	105	0.848	297	0.574	149	0.597	230	0.856
	GCV	0	5	33	27	7	36	n.a.	36	n.a.	36	20	9
9	1	67	0.170	48	0.415	23	0.805	226	0.814	87	0.774	13	0.745
	2	78	0.249	37	0.198	23	0.741	63	0.086	69	0.917	47	0.824
	3	77	0.379	39	0.450	22	1.227	96	0.302	69	0.792	33	0.516
	GM	74	0.252	41	0.333	23	0.901	111	0.276	75	0.825	27	0.682
	GCV	8	42	14	48	3	28	73	160	14	9	73	25

n.c. = not calculated. n.a. = not applicable. INF = infinity.

		TdG		TdH		TdJ		TdK		TdL		TdM	
Lab	**Test**	**B**	**S**	**B**	**S**	**B**	**S**	**B**	**S**	**B**	**S**	**B**	**S**
10	1	n.c.	0.406	89	0.416	72	0.980	138	0.406	119	0.404	156	0.681
	2	328	0.812	107	0.852	44	0.664	160	0.437	190	0.812	127	0.818
	3	n.c.	0.873	77	0.646	55	0.730	215	0.419	74	0.224	223	1.486
	GM	328	0.660	90	0.612	56	0.780	168	0.420	119	0.419	164	0.939
	GCV	n.a.	44	17	37	25	21	23	4	50	72	29	43
11	1	57	0.289	25	0.833	17	0.992	59	0.383	47	0.996	29	1.246
	2	57	0.102	17	0.575	17	0.992	69	0.425	27	0.546	17	1.395
	3	135	0.506	17	0.387	17	0.381	72	0.225	34	0.767	34	0.222
	GM	76	0.246	19	0.570	17	0.721	66	0.332	35	0.747	25	0.728
	GCV	53	96.595	22	39.741	3	59.724	11	35.097	29	30.799	38	137.262
Overall GM		**104**	**–**	**46**	**–**	**36**	**–**	**125**	**–**	**72**	**–**	**77**	**–**
Inter-lab GCV		**84**	**–**	**61**	**–**	**51**	**–**	**58**	**–**	**67**	**–**	**77**	**–**
Median intra-lab GCV		**35**	**–**	**20**	**–**	**13**	**–**	**25**	**–**	**26**	**–**	**24**	**–**

n.c. = not calculated. n.a. = not applicable. INF = infinity.

Table 11 shows that laboratories 2, 7, 8 and 10 performed all valid assays on 3 different days, each assay including all toxoids and the detecting toxin test. Laboratory 3 carried out the tests on 6 different days but did not systematically include the test for detecting toxin on each day. To compensate for this omission the laboratory also provided data from 3 VI tests carried out during the preliminary testing phase. In addition, laboratory 3 provided readings of most tests before the application of isopropanol and after the application of isopropanol, coded in the remainder of this report as laboratory 3a and 3b respectively. Laboratories 4 and 6 split the toxoids into 2 groups of 3 and tested these on 6 different days, each day also including the detecting toxin test. Laboratory 6 retested toxoid TdJ on 2 additional days, including also the detecting toxin test. Laboratories 5 and 11 performed the tests on 7 different days, each day including also the detecting toxin. Laboratory 9 performed the tests on 4 different days, each day including all toxoids and the detecting toxin test. The reason to perform a 4th assay was that the 3rd assay for TdH was invalid and the laboratory interpreted the protocol as making the whole assay, including all other toxoids, invalid. It appeared, however, that the 1st assay had a markedly lower sensitivity and generally very different readings from those in assays 2 to 4. It was therefore decided to regard assay 1 as part of the learning phase and include only assays 2 to 4 for further analysis, with the exception of TdH for which only 2 assays were included in the analysis. Overall, the incidence of invalid assays is 8/193 or about 4 %.

The definition of the TCP in mice cannot be transferred directly to the assay on Vero cells. See Appendix 6 for a detailed explanation on the fundamental impossibility to establish *in vitro* endpoints for the prediction of death/survival in mice. It was therefore necessary to correlate *in vivo* with *in vitro* results in a different way than originally foreseen. The most logical approach seemed to be to reduce the observed responses to the underlying physical quantities as detailed below.

Assuming that N and S are known it is possible to calculate, per row, the Binding Power (B), which is the amount of antitoxin bound by the toxoid. Let us assume that 0.5 mL of a 1/60 diluted toxoid (i.e. 120 TCP units) is added to 2 IU/0.5 mL antitoxin. After incubation 1 mL of 1/170 diluted detecting toxin is added and again allowed to incubate. This 2 mL mix is further diluted 1/16 with buffer solution and 0.1 mL is loaded into the first well of the first row of the plate, with further 2-fold dilutions across that row. We assume that N = 284 IU/mL. Let us further assume that the observed S in the VI test was 0.50 nL/well and that the first 3 wells of the row show lethality. The 3rd well is therefore estimated to contain $2^{½}$ × 0.50 nL = 0.71 nL toxin and the 1st well 2.83 nL. The original tube must therefore have contained 2.83 × 20 × 16 = 905 nL toxin. The original amount of toxin added was 1 mL/170 = 5882 nL so 4977 nL must have been neutralised, for which 1.413 IU antitoxin is required. The missing 0.587 IU must have been bound by the toxoid which therefore has a binding power of 0.587 IU/0.5 mL at 1/60 dilution or B = 70 IU/mL. This

method can be applied to each row individually or to the plate as a whole by ML methods which optimise the parameters of interest for all rows simultaneously. In this report ML estimators are used because they have the advantage that rows with 100 % lethality or survival can be taken into account whereas this is not possible for individual rows. More details on the ML method used are given in Appendix 5.

The calculation method depends on assumptions about the true values of N and S. The assumed N = 284 IU/mL is thought to be fairly accurate as it is based on consensus and can reasonably be assumed to be constant across laboratories. The assumed value for S, however, is not only different per laboratory, but it is also based on the outcome of only a small number of VI tests, in most cases only 3 per laboratory. To demonstrate the relevance of accurate assumptions on S, the TCP values were estimated in 3 different ways:

1. without prior information on S (i.e. S and B are estimated simultaneously for each individual test);
2. with prior information from the VI test carried out on the same day (i.e. S is first estimated from the VI test run in parallel and then kept fixed to estimate B for an individual test);
3. with prior information from the pooled VI test carried out by that laboratory (i.e. the data of all VI tests are pooled, yielding an estimate for S which is then kept fixed for all TCP tests carried out by that same laboratory).

The results are listed in Tables 12, 13 and 14 for each of the 3 methods respectively. The data are also shown graphically in Figures 6, 7 and 8.

Table 13 – *Estimated TCP values (IU/mL) using sensitivity obtained with parallel VI test*

		TdG		TdH		TdJ		TdK		TdL		TdM	
Lab	Test	B	S	B	S	B	S	B	S	B	S	B	S
2	1	n.c.	0.164	41	0.164	32	0.164	129	0.164	79	0.164	54	0.164
	2	157	0.348	60	0.348	33	0.348	165	0.348	79	0.348	56	0.348
	3	90	0.466	56	0.466	33	0.466	126	0.466	65	0.466	40	0.466
	GM	119	0.299	52	0.299	33	0.299	139	0.299	74	0.299	49	0.299
	GCV	41	58	20	58	2	58	15	58	11	58	19	58
3a	1	122	0.134	46	0.270	40	0.270	173	0.270	88	0.270	101	0.270
	2	276	0.270	71	0.270	35	0.081	123	0.081	72	0.270	67	0.270
	3	216	0.270	82	0.270	30	0.081	n.c.	0.269	100	0.269	129	0.269
	GM	194	0.214	64	0.270	35	0.121	146	0.180	86	0.270	95	0.270
	GCV	44	42	31	0	15	79	24	79	17	0	34	0
3b	1	83	0.270	30	0.270	38	0.270	173	0.270	88	0.270	62	0.270
	2	186	0.270	71	0.270	43	0.134	169	0.134	65	0.270	67	0.270
	3	201	0.270	64	0.270	30	0.134	179	0.761	97	0.761	57	0.761
	GM	146	0.270	51	0.270	37	0.169	173	0.302	82	0.381	62	0.381
	GCV	52	0	50	0	18	42	3	107	21	66	8	66
4	1	126	0.502	47	0.662	30	0.662	149	0.502	76	0.662	44	0.502
	2	145	0.576	58	0.408	35	0.408	179	0.576	71	0.408	54	0.576
	3	190	0.469	51	0.618	39	0.618	170	0.469	81	0.618	62	0.469
	GM	151	0.514	52	0.551	34	0.551	165	0.514	76	0.551	53	0.514
	GCV	21	11	11	27	13	27	9	11	6	27	17	11
5	1	75	0.212	27	0.184	10	0.184	97	0.212	30	0.140	50	0.198
	2	49	0.212	26	0.212	18	0.212	77	0.140	41	0.140	36	0.140
	3	46	0.140	18	0.212	7	0.212	90	0.198	46	0.198	64	0.198
	GM	55	0.184	23	0.202	11	0.202	88	0.180	38	0.157	48	0.176
	GCV	27	24	25	8	48	8	12	23	22	20	30	20

n.c. = not calculated.

		TdG		TdH		TdJ		TdK		TdL		TdM	
Lab	**Test**	**B**	**S**	**B**	**S**	**B**	**S**	**B**	**S**	**B**	**S**	**B**	**S**
6	1	192	0.128	59	0.128	27	0.388	215	0.388	95	0.388	96	0.388
	2	268	0.512	91	0.512	27	0.194	264	0.676	120	0.676	126	0.676
	3	168	0.388	45	0.388	27	0.194	230	0.274	n.c.	0.274	165	0.274
	GM	205	0.294	62	0.294	27	0.245	235	0.416	107	0.416	126	0.416
	GCV	24	84	37	84	0	42	11	48	16	48	28	48
7	1	151	0.469	49	0.469	27	0.469	165	0.469	74	0.469	64	0.469
	2	172	0.538	57	0.538	29	0.538	201	0.538	95	0.538	69	0.538
	3	181	0.576	54	0.576	30	0.576	201	0.576	92	0.576	72	0.576
	GM	168	0.526	53	0.526	29	0.526	188	0.526	87	0.526	68	0.526
	GCV	10	11	8	11	5	11	11	11	14	11	6	11
8	1	108	0.164	66	0.164	48	0.164	162	0.164	72	0.164	97	0.164
	2	n.c.	0.466	111	0.466	70	0.466	206	0.466	103	0.466	162	0.466
	3	193	0.208	87	0.208	39	0.208	137	0.208	58	0.208	84	0.208
	GM	144	0.252	86	0.252	51	0.252	166	0.252	75	0.252	110	0.252
	GCV	43	59	27	59	30	59	21	59	30	59	36	59
9	1	149	0.661	64	0.661	32	0.661	192	0.661	77	0.661	13	0.661
	2	148	0.709	71	0.709	34	0.709	64	0.709	60	0.709	38	0.709
	3	154	1.075	59	1.075	29	1.075	211	1.075	81	1.075	55	1.075
	GM	150	0.796	65	0.796	32	0.796	137	0.796	72	0.796	30	0.796
	GCV	2	27	9	27	8	27	75	27	17	27	85	27
10	1	n.c.	0.381	84	0.381	42	0.381	131	0.381	114	0.381	78	0.381
	2	196	0.469	71	0.469	39	0.469	168	0.469	109	0.469	86	0.469
	3	258	0.502	66	0.502	46	0.502	244	0.502	113	0.502	73	0.502
	GM	225	0.447	73	0.447	42	0.447	175	0.447	112	0.447	79	0.447
	GCV	20	14	12	14	8	14	32	14	2	14	8	14
11	1	88	2.475	35	2.475	22	2.475	102	2.475	80	2.475	36	2.475
	2	107	2.153	24	2.153	21	2.153	150	2.153	39	2.153	19	2.153
	3	n.c.	2.654	27	2.654	25	2.654	268	2.654	51	2.654	95	2.654
	GM	97	2.418	28	2.418	23	2.418	160	2.418	54	2.418	40	2.418
	GCV	14	10.684	19	10.684	10	10.684	52	10.684	37	10.684	96	10.684
Overall GM		**142**	**–**	**52**	**–**	**30**	**–**	**157**	**–**	**76**	**–**	**63**	**–**
Inter-lab GCV		**41**	**–**	**40**	**–**	**42**	**–**	**25**	**–**	**30**	**–**	**46**	**–**
Median intra-lab GCV		**24**	**–**	**20**	**–**	**10**	**–**	**15**	**–**	**17**	**–**	**28**	**–**

n.c. = not calculated.

Table 14 – *Estimated TCP values (IU/mL) using sensitivity obtained with combined VI test per laboratory*

		TdG		TdH		TdJ		TdK		TdL		TdM	
Lab	Test	B	S	B	S	B	S	B	S	B	S	B	S
2	1	n.c.	0.314	53	0.314	34	0.314	202	0.314	125	0.314	81	0.314
	2	145	0.314	57	0.314	33	0.314	155	0.314	74	0.314	53	0.314
	3	72	0.314	47	0.314	32	0.314	99	0.314	49	0.314	36	0.314
	GM	102	0.314	52	0.314	33	0.314	146	0.314	77	0.314	54	0.314
	GCV	53	0	9	0	3	0	37	0	49	0	42	0
3a	1	198	0.270	46	0.270	40	0.270	173	0.270	88	0.270	101	0.270
	2	276	0.270	71	0.270	61	0.270	279	0.270	72	0.270	67	0.270
	3	216	0.270	82	0.270	42	0.270	n.c.	0.270	100	0.270	130	0.270
	GM	227	0.270	64	0.270	47	0.270	219	0.270	86	0.270	95	0.270
	GCV	17	0	31	0	24	0	35	0	17	0	34	0
3b	1	83	0.270	30	0.270	38	0.270	173	0.270	88	0.270	62	0.270
	2	186	0.270	71	0.270	63	0.270	280	0.270	65	0.270	67	0.270
	3	201	0.270	64	0.270	35	0.270	95	0.270	59	0.270	40	0.270
	GM	146	0.270	51	0.270	44	0.270	166	0.270	70	0.270	55	0.270
	GCV	52	0	50	0	32	0	58	0	21	0	29	0
4	1	131	0.532	42	0.532	28	0.532	154	0.532	67	0.532	45	0.532
	2	137	0.532	71	0.532	37	0.532	169	0.532	88	0.532	52	0.532
	3	200	0.532	48	0.532	37	0.532	186	0.532	72	0.532	66	0.532
	GM	153	0.532	52	0.532	34	0.532	169	0.532	75	0.532	54	0.532
	GCV	24	0	27	0	16	0	9	0	14	0	19	0
5	1	56	0.181	27	0.181	10	0.181	81	0.181	41	0.181	45	0.181
	2	41	0.181	22	0.181	14	0.181	103	0.181	56	0.181	49	0.181
	3	71	0.181	14	0.181	5	0.181	81	0.181	41	0.181	53	0.181
	GM	55	0.181	20	0.181	9	0.181	88	0.181	45	0.181	49	0.181
	GCV	28	0	33	0	50	0	14	0	18	0	9	0
6	1	n.c.	0.302	140	0.302	21	0.302	168	0.302	75	0.302	75	0.302
	2	150	0.302	50	0.302	41	0.302	119	0.302	57	0.302	58	0.302
	3	131	0.302	35	0.302	41	0.302	253	0.302	n.c.	0.302	182	0.302
	GM	140	0.302	63	0.302	33	0.302	172	0.302	65	0.302	93	0.302
	GCV	10	0	82	0	41	0	39	0	19	0	66	0
7	1	169	0.526	52	0.526	29	0.526	179	0.526	87	0.526	68	0.526
	2	169	0.526	56	0.526	29	0.526	198	0.526	94	0.526	68	0.526
	3	169	0.526	52	0.526	29	0.526	188	0.526	87	0.526	68	0.526
	GM	169	0.526	53	0.526	29	0.526	188	0.526	89	0.526	68	0.526
	GCV	0	0	5	0	0	0	5	0	5	0	0	0
8	1	179	0.267	95	0.267	60	0.267	228	0.267	128	0.267	125	0.267
	2	179	0.267	72	0.267	52	0.267	142	0.267	64	0.267	114	0.267
	3	n.c.	0.267	104	0.267	43	0.267	161	0.267	66	0.267	93	0.267
	GM	179	0.267	89	0.267	51	0.267	173	0.267	82	0.267	110	0.267
	GCV	0	0	19	0	17	0	25	0	41	0	15	0
9	1	162	0.797	73	0.797	36	0.797	222	0.797	89	0.797	13	0.797
	2	162	0.797	77	0.797	36	0.797	75	0.797	64	0.797	46	0.797
	3	124	0.797	50	0.797	25	0.797	169	0.797	69	0.797	45	0.797
	GM	148	0.797	65	0.797	32	0.797	141	0.797	73	0.797	30	0.797
	GCV	16	0	24	0	21	0	61	0	18	0	82	0

n.c. = not calculated.

		TdG		TdH		TdJ		TdK		TdL		TdM	
Lab	**Test**	**B**	**S**	**B**	**S**	**B**	**S**	**B**	**S**	**B**	**S**	**B**	**S**
10	1	n.c.	0.447	94	0.447	45	0.447	147	0.447	129	0.447	87	0.447
	2	189	0.447	69	0.447	39	0.447	162	0.447	105	0.447	83	0.447
	3	237	0.447	63	0.447	43	0.447	225	0.447	105	0.447	69	0.447
	GM	212	0.447	74	0.447	42	0.447	175	0.447	112	0.447	79	0.447
	GCV	16	0	22	0	8	0	23	0	12	0	13	0
11	1	87	2.402	35	2.402	22	2.402	100	2.402	79	2.402	35	2.402
	2	115	2.402	26	2.402	22	2.402	163	2.402	40	2.402	20	2.402
	3	n.c.	2.402	26	2.402	24	2.402	248	2.402	48	2.402	88	2.402
	GM	100	2.402	28	2.402	23	2.402	159	2.402	53	2.402	40	2.402
	GCV	20	0	17	0	6	0	48	0	36	0	86	0
Overall GM		**139**	**–**	**52**	**–**	**32**	**–**	**160**	**–**	**73**	**–**	**62**	**–**
Inter-lab GCV		**42**	**–**	**45**	**–**	**51**	**–**	**23**	**–**	**25**	**–**	**41**	**–**
Median intra-lab GCV		**17**	**–**	**24**	**–**	**17**	**–**	**35**	**–**	**18**	**–**	**29**	**–**

n.c. = not calculated.

Figure 6 – *Scatter plot of TCP results (in vitro) per laboratory and per toxoid without prior information on sensitivity of Vero cells*

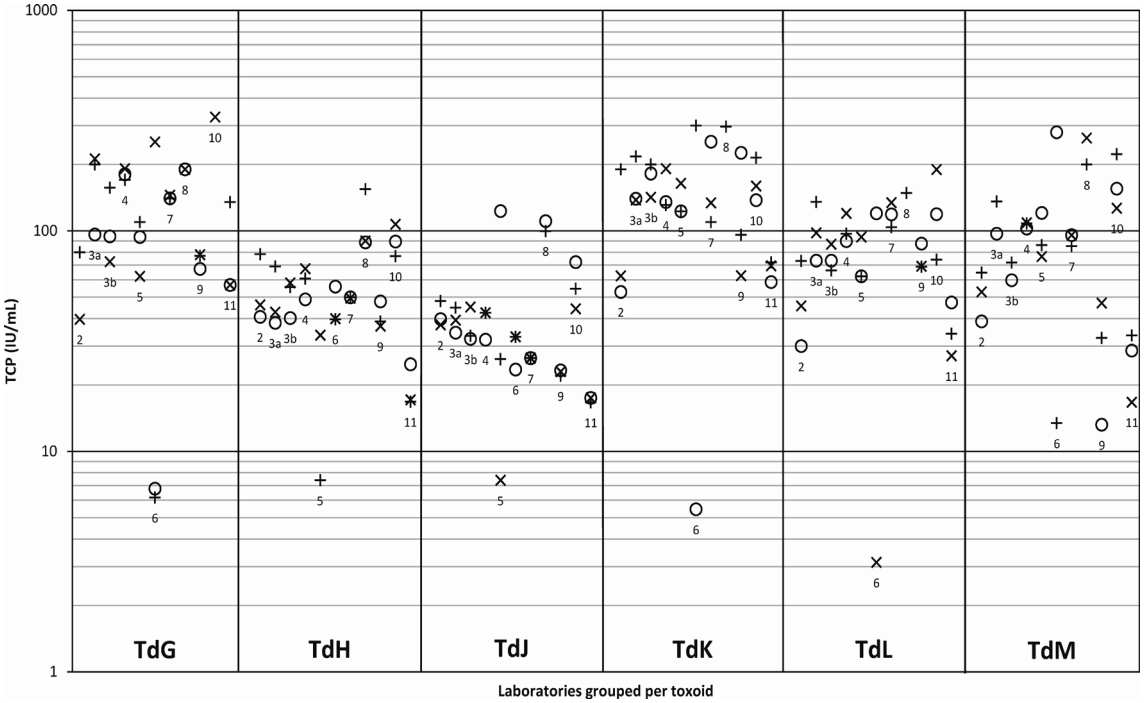


Figure 7 – *Scatter plot of TCP results (in vitro) per laboratory and per toxoid using sensitivity obtained with parallel VI test*

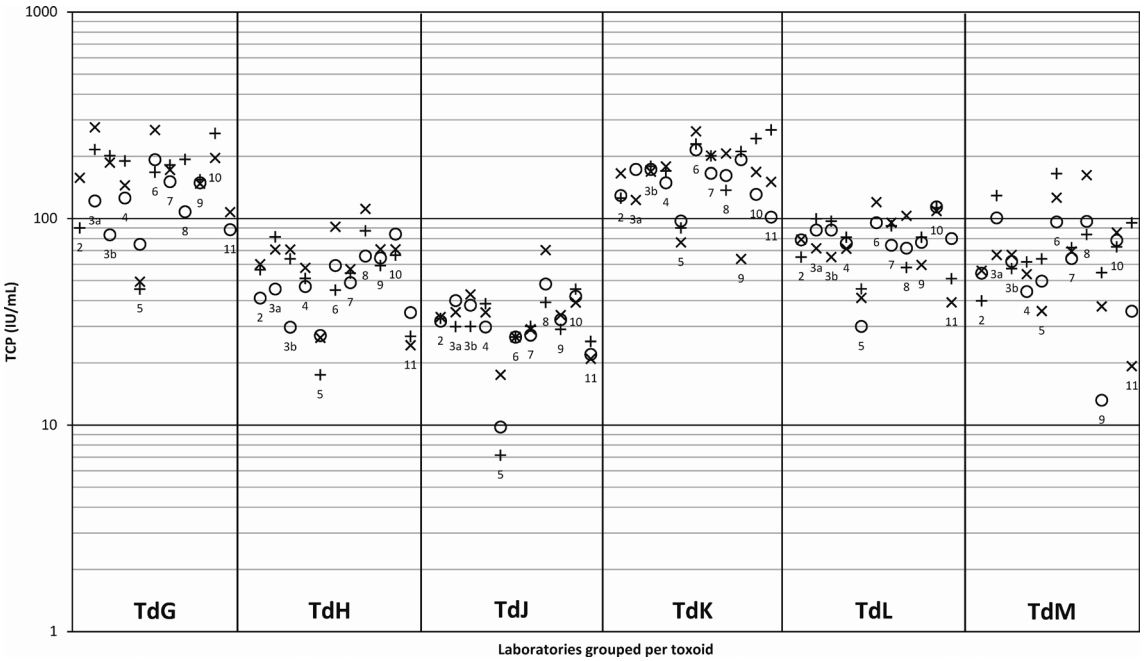
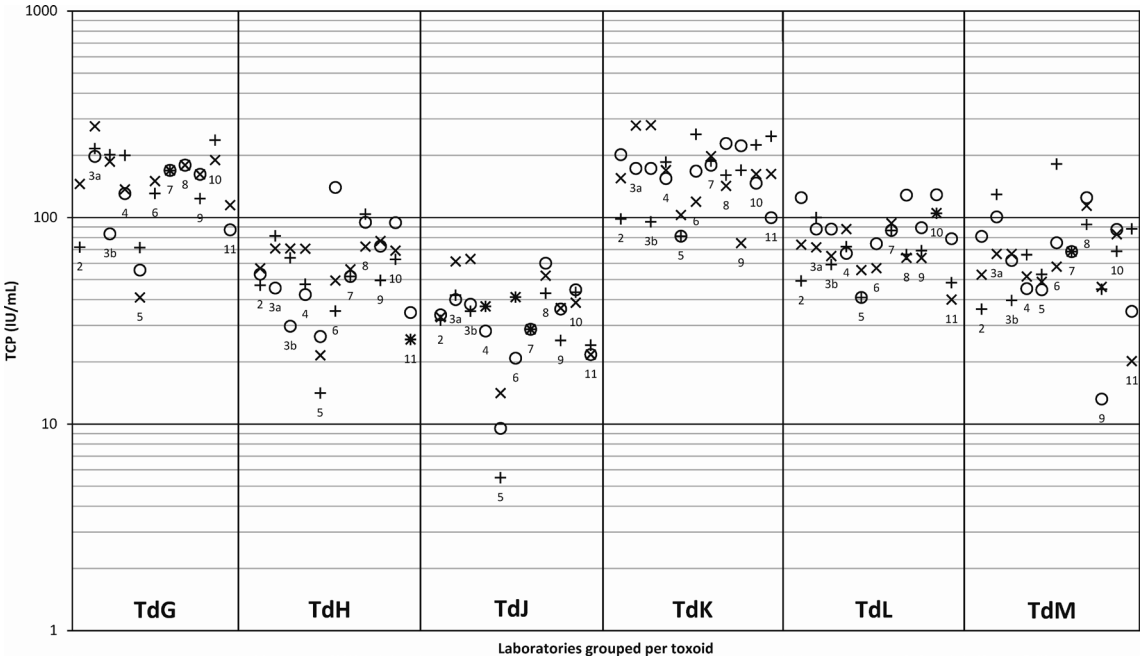


Figure 8 – *Scatter plot of TCP results (in vitro) per laboratory and per toxoid using sensitivity obtained with combined VI tests per lab*



The first method appears to be rather unstable as it does not always converge to the same solution if the optimisation algorithm is started at different starting values. Table 12 shows only the results where unambiguous convergence was obtained. However it can be seen that the estimates for S and B sometimes vary beyond reasonable boundaries with this approach. This demonstrates the necessity for reliable prior information on S or the need to design a plate lay-out which allows for accurate estimation of S for individual plates. The 2nd and 3rd methods give very similar overall results but reproducibility appears to be slightly better when sensitivity is estimated from the parallel VI tests instead of a pooled VI tests. This would imply that it is better to include information on sensitivity in the design of the TCP assay itself than to establish a 'validated' sensitivity. Several alternative designs are discussed in Appendix 6.

Comparisons between the MLD assays in mice and on Vero cells

For laboratories having carried out both methods it is possible to calculate the average LD50 per test toxin in both methods and their ratio. The ratio within any given laboratory should not vary more than 2 dilution steps of the least precise method, in this case a factor 9 because the mouse assay was performed in 3-fold steps. Table 15 shows the results of this comparison.

The table shows that laboratories 2 and 3 have a rather consistent ratio for all toxins with less than a factor 2 between any pair of ratios, although the ratios of the test toxins in laboratory 2 are generally lower than that of the CSTx. Laboratory 4, however, obtained markedly higher ratios for TxE and TxF which would mean that these toxins do not behave in a similar way in both methods. Laboratory 5 obtained ratios generally lower than for CSTx but within the 9-fold range. Laboratory 6 obtained ratios within a factor 2.5 from the CSTx but it should be recalled that the 3-fold CSTx assay seemed to reveal instability of the material so this comparison may not be very meaningful.

Another way to compare the methods is by graphical assessment of the ranking. Figure 9 shows in the left half the average result per laboratory and per toxin for the mouse assays and in the right half for the Vero cell assays. All values are with respect to the MLD of the CSTx in the relevant assay. The toxins are connected between laboratories by straight lines. Numbers below the plots are the laboratory codes.

The figure shows that both methods achieve a clear separation between the lowest toxins TxC and TxD. Discrimination between the other 4 toxins is less clear because they are of similar toxicity but there is a weak indication that a slightly better discrimination is achieved with the Vero cell assay. Overall, all results are in the same order of magnitude with both methods.

Figure 10 shows the same results but this time as rank, giving rank 1 to the toxin with lowest MLD and rank 6 to the one with highest MLD. This plot shows more clearly the reproducible separation between TxC and TxD. The improved discrimination between the other 4 toxins can be seen because TxB is mostly ranked 3 or 4 with the Vero cell assay (except by laboratory 2) whereas this toxin is found in all ranks from 3 to highest with the mouse assay. A similar observation can be made for TxE. The only marked inversion for the Vero cell assay is observed for TxF in laboratories 9 and 10.

Table 15 – *Ratios of sensitivity per toxin (in vivo/in vitro)*

Lab	CSTx	TxA	TxB	TxC	TxD	TxE	TxF
2	1450	1150	1020	650	600	1050	1170
3	760	590	620	680	910	720	910
4	880	950	1050	600	2040	7590	10,470
5	2930	1480	710	1150	2820	950	1820
6	820	1050	470	350	1780	850	1350

Figure 9 – *Comparison of ranking in vivo and in vitro MLD results (with respect to CSTx)*

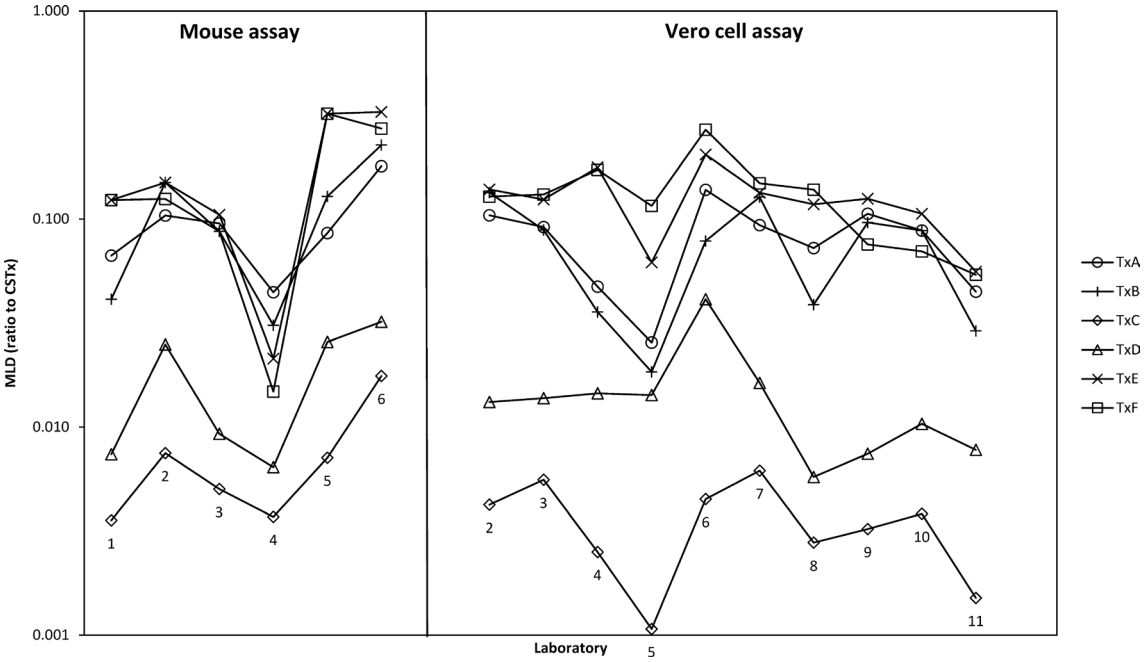
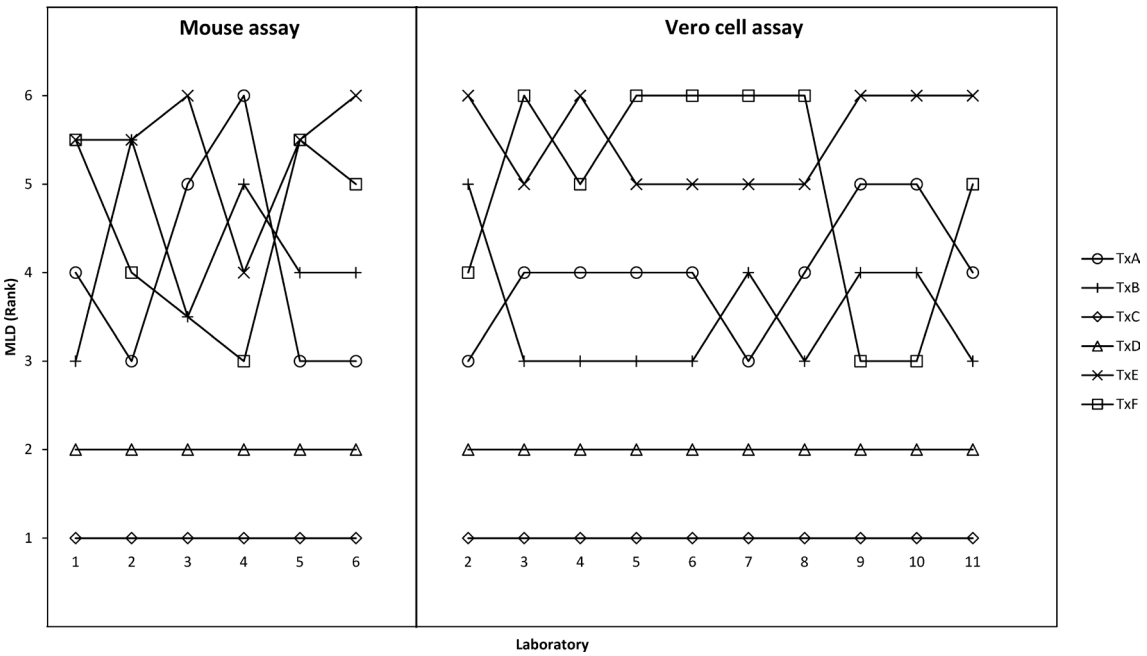


Figure 10 – *Comparison of ranking in vivo and in vitro MLD results*



Comparisons between the TCP assays in mice and on Vero cells

Ranking of results can also be done for the TCP assays in a similar way as for the MLD assays. Figure 11 shows that both methods achieve a rather clear separation between the lowest toxoid TdJ and the other toxoids, the only exception being laboratory 9 which found TdM lower than all other toxoids in the Vero cell assay. Apart from the markedly lower values obtained by laboratory 5 the results appear reproducible and of similar magnitude as the mouse results.

Figure 11 – *Comparison of ranking in vivo and in vitro results*

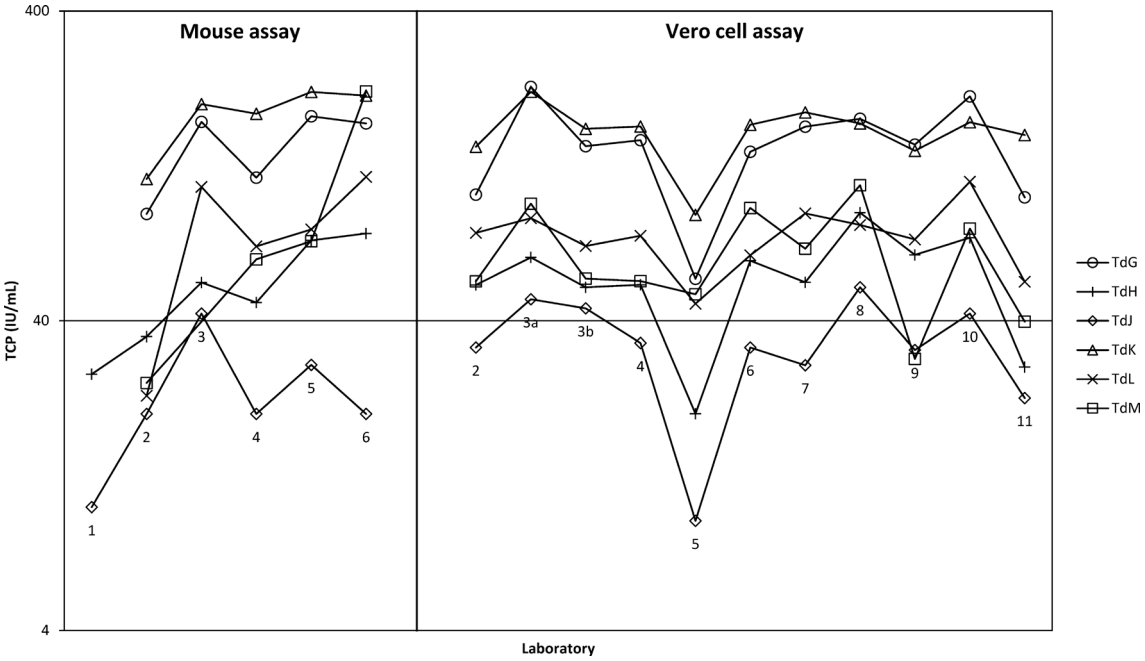


Figure 12 – *Comparison of ranking in vivo and in vitro results*

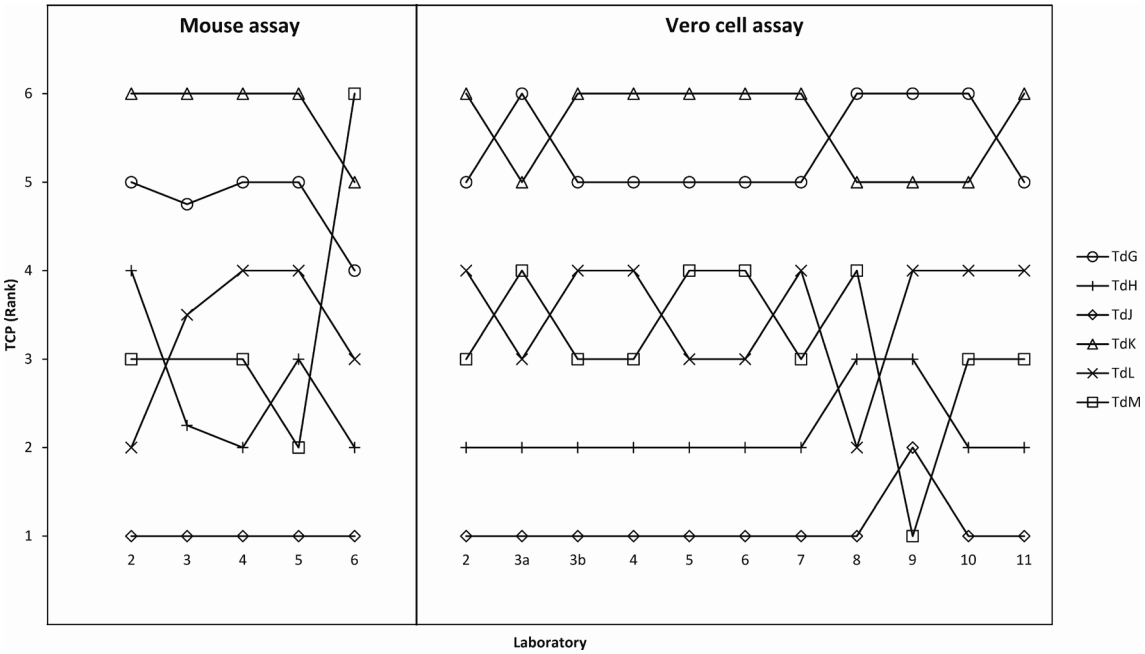


Figure 12 shows more clearly that ranking is fairly consistent across laboratories with both methods. In the Vero cell assay TdK and TdG are ranked 5 or 6 by all laboratories, whereas laboratory 6 found TdM highest in the mouse assay. In the Vero cell assay TdL and TdM are mostly ranked 3 or 4 with the exception of laboratories 8 and 9.

Concordance correlation between *in vivo* and *in vitro* methods

The overall averages per toxin and per toxoid for the relevant methods are summarised in Table 16 and plots are shown in Figures 13 and 14. The *in vitro* results for TCP are with respect to the VI test carried out in parallel (method 2). The results for MLD are shown on a logarithmic

scale due to the geometric nature of the dilution steps whereas results for TCP are shown on a linear scale due to the arithmetic progression of the doses. The diagonal line is the line of perfect agreement. The closer the dots are to this line, the better the concordance.

Lin's concordance correlation between the MLD methods is ρ_c = 0.961 (using log-transformed values) and ρ_c = 0.921 (using non log-transformed values). Lin's concordance correlation between the TCP methods is ρ_c = 0.968 (using log-transformed values) and ρ_c = 0.980 (using non log-transformed values).

6. DISCUSSION AND CONCLUSIONS

The mice used in the 6 laboratories performing the *in vivo* testing showed a variation in sensitivity to the detecting toxin of greater than 12-fold. However, if the outlier value from laboratory 5 was removed this sensitivity range was reduced to just over 6-fold. As expected, all of the participants' Vero cell lines were far more sensitive to the lethal effects of C. septicum toxin than any of the mouse strains. In most cases the Vero cells were almost 1 000 times more sensitive than the participants' relevant mouse strain, demonstrating the potentially greater sensitivity of the cell line assays. The Vero cells used in the 10 laboratories performing the *in vitro* testing showed a toxin sensitivity range of approximately 24-fold but if the outlier values from laboratories 9 and 11 were removed the range was reduced to just over 3-fold. It is not possible to generally, across all 10 laboratories, equate cell well deaths to mouse deaths. However, it was found that for each individual laboratory it may be possible to define a threshold where the number of dead cell wells translates to a prediction as to whether a mouse would have died at a specific toxin dose. In our opinion, the Vero cell assay data should not be expressed in this manner as they are replacement assays not merely substitutions.

When tested for latent toxicity, at 5 IU/ml, the standard antitoxin (VI) showed no toxicity in Vero cells in any of the laboratories. Therefore, the presence of antitoxin in the final TCP mixtures that were applied to the Vero cells would not have had any interfering effect on the assay outcomes. In contrast all of the toxoids exhibited some latent Vero cell toxicity in most of the participating laboratories. This was to be expected as all of the toxoids would have had their toxoiding protocol validated by a mouse test, which means that due to the greater sensitivity of the Vero cell assays the toxoids could still be expected to be cytotoxic even after a 1 in 10 dilution.

There was a certain amount of variation between the laboratories with regard to the level of Vero cell toxicity associated with the toxoids. Laboratory 6 generally showed the highest level of cell death even though this laboratory's cell line was not the most sensitive to the *C. septicum* detecting toxin (CSTx). In contrast, laboratory 4 showed the lowest level of cell death despite not using the least sensitive Vero cells. There were also clear differences between the levels of the toxic effects of the sample toxoids. Overall toxoid TdG was found to have the greatest latent toxicity by eight of the 10 laboratories with toxoid TdK having the lowest latent toxicity in 6 of the laboratories. These results could mean that there were other Vero cell toxic components present in the toxoids. These toxic components could be untoxoided minor toxins or even residual toxoiding chemicals such as formaldehyde. However, as these toxoids, when assessed in the Vero cell TCP assay, were applied to the Vero cell wells at final concentrations far below those used in the latent toxicity testing any residual toxicity effects were unlikely to have any bearing on the assay outcomes.

The preliminary ranging assays were to determine the optimal dose range for the toxins and toxoids and to assess whether the suggested L^+ value, of 1/170, for the CSTx challenge toxin was suitable for the mice used in the *in vivo* TCP tests. This L^+ value was applicable for laboratories 1 to 4 but laboratories 5 and 6, using mice which were the least sensitive to CSTx, established lower L^+ values. The lower sensitivity of their mice probably directly contributed to the reduced L^+ values that laboratories 5 and 6 obtained.

The overall ranking of the toxins in the mouse MLD test was generally similar in all of the laboratories. This ranking ranged from TxC as the least toxic in all of the laboratories up to TxE and TxF as the most toxic in all laboratories. The inter-laboratory GCVs for these assays ranged

from 69 % to 117 %, which is reasonably good for such an animal-based test. The ranking of the toxins in the Vero cell MLD assay was again similar in all of the laboratories and similar to that seen in the mouse MLD tests. TxC was again found to be the least toxic in all of the laboratories and TxE and TxF were the most toxic. The inter-laboratory GCVs ranged from 143 % to 183 % but when corrected for each laboratory's Vero cell sensitivity to CSTx the range was reduced to 43 % to 77 % which is very good. The incidence of reported invalid Vero cell MLD assays was approximately 9 % which is acceptably low for a routine assay and remarkably good for a new form of assay with which the participants were unfamiliar. However, it must be borne in mind that, although they were requested to do so, some of the participants may not have reported invalid assays generated during their initial familiarisation with the assay.

The toxin/antitoxin test allowed quantification of the toxin equivalence of the detecting toxin (CSTx) in combination with the sensitivity of the relevant Vero cell line. Using this approach the inter-laboratory GCVs were very low at only 7 %. This method could be a very useful way of expressing the toxicity of different toxins in terms of an appropriate standard antitoxin. Such an approach could be used to allow direct comparisons of the same type of toxin from a variety of different sources with greatly improved accuracy and reproducibility.

The TCP assay in mice generally ranked the toxoids in a similar order in most of the laboratories with TdJ having the lowest value and TdK the highest. The TCP values in mice cannot be directly transferred to the Vero cell assay. However, the Vero cell assay also tended to rank the toxoids in a similar order to the mouse test with TdJ as the lowest and TdK as the highest. Once again the level of invalid Vero cell assays was remarkably low at only 4 %.

Table 16 – *Summary of overall average per method and per test material*

Toxins	Toxicity relative to CSTx	
	MLD *in vivo*	MLD *in vitro*
TxA	0.088	0.073
TxB	0.089	0.061
TxC	0.006	0.003
TxD	0.014	0.012
TxE	0.153	0.116
TxF	0.132	0.118

Toxoids	TCP (IU/mL) *in vivo*	TCP (IU/mL) *in vitro*
TdG	142	142
TdH	48	52
TdJ	21	30
TdK	178	157
TdL	69	76
TdM	71	63

Figure 13 – *Concordance plot of the average MLD (in vitro versus in vivo)*

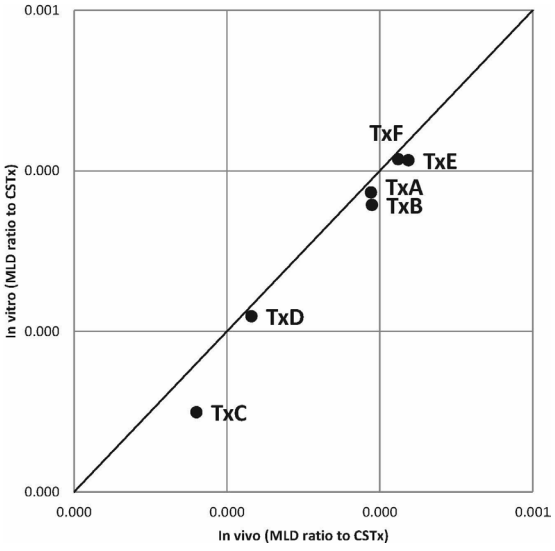
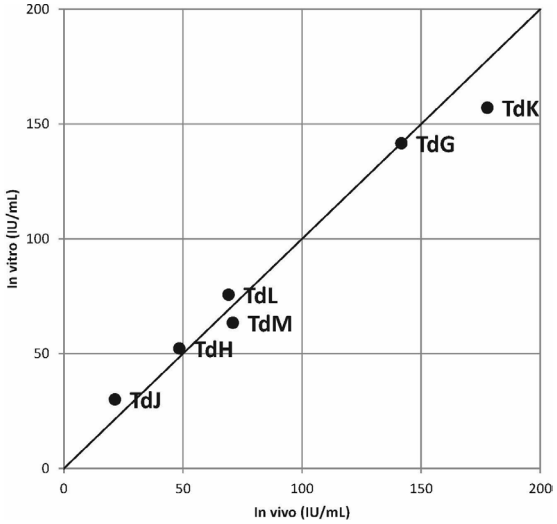


Figure 14 – *Concordance plot of the average TCP (in vitro versus in vivo)*



The results of this study demonstrate the transferability of the cell line assays. All but 1 of the participants were unfamiliar with these assays at the start of the study and 1 laboratory was even unfamiliar with the use of cell lines altogether. Yet all of the 10 laboratories involved in the *in vitro* testing were able to use these assays to obtain repeatable results with low levels of invalid assays. Reproducibility of the assays between the laboratories was good and was improved by normalisation of the MLD value expressed as a ratio to the detecting toxin and when the TCP results were expressed in relation to antitoxin activity neutralised. The development of improved statistical methods during the course of the study allowed more information to be extracted from the results of the Vero cell assays than from the corresponding *in vivo* tests. The fact that antigen quantification was better characterised by TCP assays on Vero cells than in mice has advantages for the more accurate formulation of vaccines, thereby generating savings and more consistent final products.

Comparison of the *in vivo* MLD test with the Vero cell method showed that both clearly distinguished between the least toxic toxins (TxC and TxD) and between them and the other toxins. Neither method gave a truly clear separation between the 4 other toxins, which were of similar toxicity, but there was a slightly better discrimination using the Vero cell assay. When the toxins were ranked according to the results from the different laboratories there was improved discrimination and again the Vero cell assay gave the clearer separation. When ranking was applied to the TCP assay results both the *in vivo* and *in vitro* assays distinguished between the lowest ranked toxoid (TdJ) and the others. Apart from the values from 1 laboratory, the results appear to be reproducible and of similar magnitudes. As a consequence the ranking is fairly consistent across the laboratories with both methods.

The concordance correlations between the *in vivo* and *in vitro* methods were for the MLD assays $\rho_c = 0.961$ (using log-transformed values) and $\rho_c = 0.961 = 0.921$ (using non log-transformed values) and for the TCP assays $\rho_c = 0.961 = 0.968$ (using log-transformed values) and $\rho_c = 0.961 = 0.980$ (using non log-transformed values). These correlations are excellent allowing the proposal that the Vero cell assays can be used as alternatives to the mouse tests for the assessment of *C. septicum* toxin MLD and toxoid TCP values.

There were some minor issues with the study, most of which were linked to the protocol. Only 1 of the participating laboratories had previous experience with using these cell line assays. It had therefore been decided to retain the methodology of the *in vivo* assays, which at least 5 of the laboratories were familiar with, as much as possible up to the point where the test samples and/or mixtures were assessed for toxicity by application to the Vero cells. For the laboratories performing both the *in vivo* and *in vitro* assays this meant that they could theoretically run both types of assay simultaneously with the same final mixtures applied either to mice or Vero cells. It was subsequently discovered that the workload involved in performing both *in vivo* and *in vitro* tests simultaneously proved too great for most of the laboratories so the different assays were rarely done together. As the volumes of reagents and samples to be used in each assay, as stipulated in the protocol, were optimised for the mouse tests, and were much greater than those needed for the Vero cell assays, some of the laboratories came close to running out of materials before they could complete the full testing programme.

It had been assumed that it would be possible to do a statistical analysis allowing direct comparison of the *in vivo* and *in vitro* results. However, as the results accumulated it soon became apparent that due to the novelty of the Vero cell assays and their much greater sensitivity this would not be possible. A new approach to the statistical analysis employing maximum likelihood methods was then applied to the data. The results from this analysis have been valuable but more useful information could have been obtained if the protocol had been originally designed to optimise the collection of data from the *in vitro* assays. This is a finding that will have to be addressed in the design of any future studies of these types of assays.

During pre-study assessment of the detecting toxin (CSTx), when stored as described in the protocol, it retained its original toxicity over the required time. However, during the course of the study 2 laboratories reported results that suggested that the detecting toxin may have been losing toxicity towards the end of the testing period. The testing in some of the laboratories stretched over a much longer period than scheduled, which was also longer than the time over which the toxin had originally been assessed. It is therefore possible that over a longer storage

time the detecting toxin may have begun to lose toxicity and could have had an adverse effect on the outcomes of some of the later assays. This possibility will have to be considered and resolved for any future studies.

In conclusion, in spite of some shortcomings, this study demonstrated that the *in vitro* repeatability and reproducibility of the *in vitro* Vero cell based MLD and TCP assays are not worse than that of the *in vivo* assays. Therefore, the *in vitro* assays can replace the *in vivo* ones. They are relatively easily transferable to other laboratories which, even though unfamiliar with the methods, quickly seem to master them as demonstrated by the low levels of invalid assays. The analysis has shown that with a protocol and methodologies optimised for the *in vitro* assays it would be possible to obtain even more sensitive, accurate and reproducible results with this type of assay and not only for C. septicum toxins and toxoids but, potentially, for all clostridial antigens based on cytotoxins. Most importantly this study has demonstrated concordance between the *in vitro* and *in vivo* assays of such a level that these *in vitro* assays can now be confidently proposed as replacements for the mouse MLD and TCP tests for *C. septicum*. The use of these *in vitro* assays would not only produce significant savings in animal usage but also shorten the duration of the relevant QC testing and allow more accurate and reproducible blending of final vaccines.

7. RECOMMENDATIONS

The study outcome and follow up activity proposals for BSP130 were presented by the project leaders and discussed with the participants at an EDQM/EPAA workshop that took place in Egmond aan Zee on 15 and 16 September 2015. The minutes of the workshop were published 20 and served as a basis for the finalisation of the study report and for the elaboration of the present recommendations.

In addition to the proposal that the Vero cell based MLD and TCP assays should be promoted as replacements for the conventional mouse tests for C. septicum antigens, it is recommended that there should be a follow up study to fully exploit these *in vitro* assays. The findings of the current study suggest that with a protocol optimised for the *in vitro* assays alone, allied with modifications to the MLD and TCP assay as outlined in Appendix 6, it should be possible to establish improved assays which take full advantage of the sensitivity and accuracy of the Vero cell methods. These assays, with relevant modifications such as the selection of cell lines with appropriate toxin sensitivities, could be applied to all cytotoxin based clostridial antigens.

The proposed study would be to improve and broaden the applicability of the cell line assays and would, therefore, require only *in vitro* testing. Both the MLD and TCP assays would be modified. In the case of the MLD test, to further explore the potential of quantifying toxin by reference to a standard antitoxin. This approach, unlike MLD determination in mice, would allow consistent measurement of the toxin largely independent of the susceptibility to toxicity of the final biological detector step, Vero cells in this case. This would enable the objective assessment of different batches of toxin and their comparison. The possibility that the same general approach could be applied to other appropriate toxins would also be explored. The TCP assay will be modified to capitalise on the advantages of the cell lines to provide more accurate and reproducible assessments of toxoid antigenicity for use in the blending of more consistent and efficacious final vaccines. Once again the possibility that this approach could be applied to other appropriate toxoids would be investigated.

The measurement of neutralisation of Vero cell toxicity by antitoxin opens up an additional possibility. This would be the replacement of the second step of the conventional clostridial vaccine potency test, the assessment of toxin neutralisation in mice, by a cell line assay where appropriate.

The above recommendations, if successfully pursued, offer opportunities to significantly reduce animal usage, to shorten the duration of QC test procedures, to increase the accuracy and precision of MLD, TCP and potency assays providing more accurate and reproducible dosing of antigens in the final blended vaccines, to promote compendial acceptance and to proffer a basis for improved international harmonisation across this area of product testing.

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10. ABBREVIATIONS

B: binding power; BSP: Biological Standardisation Programme; C: Clostridium; CSTx: Clostridium septicum reference/detecting toxin; D: dead; DBO: Department of Biological Standardisation, OMCL Network & HealthCare; EC: European Commission; EDQM: European Directorate for the Quality of Medicines & HealthCare; EPAA: European Partnership for Alternative Approaches to Animal Testing; ETS: European Treaty Series; EU: European Union; GCV: geometric coefficient of variation; GM: geometric mean; INV: invalid; IS: International Standard; IU: International Unit; L: alive; Lab.: laboratory; LD50: median lethal dose; MEM: Minimum Essential Medium; ML: Maximum Likelihood; MLD: Minimum Lethal Dose; n.a.: not applicated; n.c.: not calculated; Ph. Eur.: European Pharmacopoeia; N: toxin equivalence; NBS: Nutrient Broth Saline; NC3Rs: National Centre for the Replacement Refinement & Reduction of Animals in Research; OCABR: Official Control Authority Batch Release; OD: Optical Density; OMCL: Official Medicines Control Laboratory; ρ_c: concordance correlation; 3Rs: replacement, reduction and refinement of animal assays; S: sensitivity; SOP: Standard Operating Procedure; TCP: Total Combining Power; VI: *C. septicum* standard antitoxin.

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12. APPENDICES

Appendix 1. General information

Appendix 1-1. Methods aims, principles and endpoints

Aims

During the production process, manufacturers routinely perform quality control tests to measure the freedom from toxicity of C. septicum toxoid (the MLD test) and the antigenicity of C. septicum toxoid (the TCP test): the current Ph. Eur. monograph 0364 requires in its section 2-3-1 a residual toxicity test aimed at controlling the efficacy of the toxoiding process. Currently almost all manufacturers perform MLD and TCP *in vivo* using mouse as toxicity indicator whilst Dr K. Redhead at MSD UK developed MLD and TCP *in vitro* using Vero cells as toxicity indicator 18. The present study was designed to assess the performance of *in vitro* methods, based on those originally developed at MSD, for the measurement of the freedom from toxicity of C. septicum toxoid (the MLD test) and of the antigenicity of C. septicum toxoid (the TCP test) and also for the toxicity of C. septicum toxins (the MLD test). The general principles and the endpoints of the methods used in the study are detailed thereafter.

Principles and endpoints

A. In vivo mouse tests

A. MINIMUM LETHAL DOSE (MLD)

Alpha toxin is the major potent cytotoxin produced by the bacterium C. septicum. In this assay dilutions of C. septicum supernatant are applied to groups of 2 mice, which are monitored for signs of toxicity and death for up to 4 days. Endpoints are recorded as the reciprocal of the last toxin dilution causing the death of both of the test animals within the given period.

B. TOTAL COMBINING POWER (TCP)

Alpha toxin is the major potent toxin produced by the bacterium C. septicum. Once chemically toxoided this forms an important antigenic component in Clostridial vaccines. The *in vivo* TCP assay is used to measure the antigenicity of C. septicum alpha toxoid. Dilutions of toxoid sample are mixed and incubated with a known concentration of neutralising antiserum and then a detector toxin. The mixture is then applied to two mice which are monitored for signs of intoxication and death up to 4 days.

A good toxoid should be able to bind all neutralising antibodies at greater dilutions leaving free detector toxin which can cause mouse death. This ability to cause mouse death should continue with increasing toxoid dilutions until a point is reached when the mice are no longer killed, this is the endpoint of the assay.

Endpoints are recorded as the greatest toxoid dilution factor that, when reacted with the set amount of standard antitoxin, left insufficient antitoxin to fully neutralise the set amount of detector toxin resulting in the death of 1 mouse but not the other or, as the arithmetic mean between the toxoid dilution factor that resulted in the death of both mice and the adjacent toxoid dilution factor that resulted in the survival of both mice.

B. In vitro methods

A. MLD *IN VITRO*

Alpha toxin is the major potent cytotoxin produced by the bacterium *C. septicum*. In this assay dilutions of *C. septicum* supernatant are applied to a microtitre plate containing confluent monolayers of Vero cells.

The alpha toxin 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 first be visualised by direct observation under the microscope and then once a valid test is confirmed, 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 direct visual observation of the results and determination of the endpoint titres. The optical density of the wells is read. By comparing the ODs of the test sample wells with those of the negative control wells, endpoint titres can be determined for the test samples. The endpoint is expressed as the greatest dilution of toxin that still causes death of more than 50 % of the cells.

B. TCP *IN VITRO*

Alpha toxin is the major potent toxin produced by the bacterium *C. septicum*. Once chemically toxoided this forms an important antigenic component in Clostridial vaccines. The cell line TCP assay is used to measure the antigenicity of *C. septicum* alpha toxoid. Dilutions of toxoid sample are mixed and incubated with a known concentration of neutralising antiserum and then a detector toxin. The mixture is then applied to a microtitre plate containing confluent monolayers of Vero cells and further incubated.

A good toxoid should be able to bind all neutralising antibodies at greater dilutions leaving free detector toxin which can cause cell death. This ability to cause cell death should continue with increasing toxoid dilutions until a point is reached when the cells are no longer killed, this is the endpoint of the assay.

The effect of the mixture on the cells can first be visualised by direct observation under the microscope and then once a valid test is confirmed, 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 direct visual observation of the results and determination of the endpoint titres/units. The OD of the wells is read. By comparing the ODs of the test sample wells with those of the negative control wells, endpoint titres can be determined for the test samples. The endpoint is expressed as the greatest dilution of toxoid that still results in the death of more than 50 % of the cells.

Appendix 1-2. Terminology and definitions

General

Accuracy: the closeness of the agreement between the accepted reference value and the mean of the repeated values found.

LD50: the statistically determined quantity of a substance that, when administered by the specified route, may be expected to cause the death of 50 % of the test animals within a given period.

Limit of detection: the lowest amount of the biologically active compound in a sample which can be detected but not necessarily quantified as an exact value.

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 variance between laboratories (collaborative studies).

Range: the interval between the upper and lower concentrations of the biologically active compound in the sample for which it has been demonstrated that the analytical procedure has a suitable level of precision and accuracy.

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.

Specificity: the ability to assess unequivocally the biologically active compound in the presence of compounds which may be expected to be present.

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

Binding Power: the amount of antitoxin bound by the toxoid expressed in IU.

Cell line endpoint titres: 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 at approximately 170L+ per mL for use as the challenge or detector toxin in the TCP assays.

Flat-bottomed microtitre plate: microtitre plate with flat-bottomed wells that is suitable for the culture of Vero cells.

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.

Laboratory: the facility at which the assays are performed (coded 1 to 12).

MLD for mice *in vivo* assays: the reciprocal of the last toxin dilution causing the death of both mice estimated by calculating the dose of toxin causing 50 % lethality (LD50), corrected by half a dilution step in order to match the last dead experimental unit in the usual definition of the MLD. The MLD was also expressed as the toxicity relative to CSTx.

Negative control: microtitre plate wells containing Vero cells which have not been treated with the detecting 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 endpoints (e.g. expressed as average number of dead wells on a row).

Sensitivity of mice and Vero cells: the MLD of the detecting toxin expressed in nL per experimental unit.

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 TxA to TxF).

Test toxoid: C. septicum toxoid samples supplied for assessment in the study (coded TdG to TdM).

Toxin/antitoxin (VI) test on Vero cells: the amount of standard antitoxin, in IU, required to completely neutralise the Vero cell toxicity of a set amount of toxin.

Toxicity relative to the detecting toxin: the ratio of the MLD of the test material to the MLD of the detecting toxin.

Toxin equivalence of the detecting toxin: the amount of antitoxin, expressed in IU/mL, required to neutralise the detecting toxin.

U-bottomed microtitre plate: low adsorption microtitre plate with U-bottomed wells that is suitable for the dilution, mixing and reacting of toxins, toxoids and antitoxin.

Appendix 2. Information on study materials specifications provided to participants

Study code	Number of containers (volume)	Material	Approximative activity*		
VI	1	Antitoxin	500 IU/ampoule		
			MLD	TCP	L+ (mL)
CSTx	14 (1 mL)	Toxin	NA	NA	1/170
TxA	6 (1 mL)	Toxin	50	NA	–
TxB	6 (1 mL)	Toxin	150	NA	–
TxC	6 (1 mL)	Toxin	10	NA	–
TxD	6 (1 mL)	Toxin	30	NA	–
TxE	6 (1 mL)	Toxin	150	NA	–
TxF	5 (3 mL)	Toxin	150	NA	–
TdG	5 (3 mL)	Toxoid	NA	100	–
TdH	5 (3 mL)	Toxoid	NA	50	–
TdJ	5 (3 mL)	Toxoid	NA	10	–
TdK	5 (3 mL)	Toxoid	NA	150	–
TdL**	5 (3 mL)	Toxoid	NA	60	–
TdM	5 (3 mL)	Toxoid	NA	60	–

* Determined at MSDAH UK, except for VI; MLD for toxins; TCP for toxoids.
** TdL is a toxoid produced from toxin TxE.

Shipment of materials

Materials donated for the study were centralised by Dr K. Redhead at MSD-UK. Shipment was organised at the end of 2013 from the MSD-UK plant (Milton Keynes) to the participants' laboratories, and costs were borne by EPAA.

Appendix 3. Methods were performed by each participating laboratory

Laboratory	*In vivo* MLD *In vivo* TCP	*In vitro* MLD *In vitro* TCP
1	+	-
2	+	+
3	+	+
4	+	+
5	+	+
6	+	+
7	-	+
8	-	+
9	-	+
10	-	+
11	-	+

Codes: + done; - not done.

- Number of laboratories performing *in vivo* and *in vitro* tests = 5
- Number of laboratories performing *in vivo* tests only = 1
- Number of laboratories performing *in vitro* tests only = 5

Appendix 4. Methodological details reported by each participant laboratory

Appendix 4.1. Mouse husbandry information for in vivo TCP and MLD

MLD assays										
Lab	Mouse strain	Mouse supplier	Mouse sex	Mouse weight range (g)	Mouse age (weeks)	Number per cage	Cage dimensions l × w × h (cm × cm × cm)	Housing temp. range (°C)	Light/dark cycle *(h/h)*	Additional treatments
1	NMRI	Charles River	Female	16-18	not reported	6-8	18 × 28 × 13	19-22	natural	–
2	BKW	B and K	Female	18-20	4	4-6	12 × 30 × 12	19-23	12/12	Environmental enrichment including tubes, boxes, etc.
3	Swiss Webster	Harlan	Female	27-32	4	10	30 × 35 × 19	21.7-23.3	12/12	Rodent enrichment
4	White mice	In-house breeding	Male	17-22	4	10	22 × 22 × 14.5	21	13/11	–
5	NMRI	Janvier	Female	16-20	3-4	4	32 × 14	21-23	12/12	–
6	NMRI	Toxi-Coop	Female	17-21	not reported	10	26 × 42 × 15	20	12/12	–

TCP assays										
Lab	Mouse strain	Mouse supplier	Mouse sex	Mouse weight range (g)	Mouse age (weeks)	Number per cage	Cage dimensions l × w × h (cm × cm × cm)	Housing temp. range (°C)	Light/dark cycle *(h/h)*	Additional treatments
1	NMRI	Charles River	Female	16-18	not reported	6-8	18 × 28 × 13	19-22	natural	–
2	BKW	B and K	Female	18-20	4	4-6	12 × 30 × 12	19-23	12/12	Environmental enrichment including tubes, boxes etc
3	Swiss Webster	Harlan	Female	11-30	10-14	10	30 × 35 × 19	21.7-23.3	12/12	Rodent enrichment
4	White mice	In-house breeding	Female	17-22	4	6-10	22 × 22 × 14,5	21	13/11	–
5	NMRI	Janvier	Female	16-20	3-4	4	32 × 14	21-23	12/12	–
6	NMRI	Toxi-Coop	Female	17-21	not reported	10	26 × 42 × 15	20	12/12	–

Appendix 4.2. Methodology information for in vivo TCP and MLD

MLD assays								
Lab	Number of mice per dose	Injection volume (mL)	Diluent	Injection route	Injection site	Inspection intervals	Inspection period (days)	Endpoint
1	2 (Compulsory)	0.5 (Compulsory)	Jensen buffer for toxins and toxoids	iv	tail vein	daily	3	death
2	2 (Compulsory)	0.5 (Compulsory)	Nutrient Broth Saline	iv	tail vein	four times a day	3	morbidity (two or more moderate signs or one severe sign)
3	2 (Compulsory)	0.5 (Compulsory)	Nutrient Broth Saline	iv	tail vein	twice a day	3	death
4	2 (Compulsory)	0.5 (Compulsory)	Saline 0.85 %	iv	tail vein	daily	4	death
5	2 (Compulsory)	0.5 (Compulsory)	Nutrient Broth Saline	iv	tail vein	twice a day	3	death or euthanasia
6	2 (Compulsory)	0.5 (Compulsory)	Saline for injection	iv	tail vein	daily	3	death

TCP assays								
Lab	Number of mice per dose	Injection volume (mL)	Diluent	Injection route	Injection site	Inspection intervals	Inspection period (days)	Endpoint
1	2 (Compulsory)	0.5 (Compulsory)	Jensen buffer for toxins, Nutrient Broth Saline for standard antitoxin	iv	tail vein	daily	3	death
2	2 (Compulsory)	0.5 (Compulsory)	Nutrient Broth Saline	iv	tail vein	four times a day	3	morbidity (two or more moderate signs or one severe sign)
3	2 (Compulsory)	0.5 (Compulsory)	Nutrient Broth Saline	iv	tail vein	twice a day	3	death
4	2 (Compulsory)	0.5 (Compulsory)	Borate Saline Buffer – peptone water	iv	tail vein	daily	3	death
5	2 (Compulsory)	0.5 (Compulsory)	Nutrient Broth Saline	iv	tail vein	twice a day	3	death or euthanasia
6	2 (Compulsory)	0.5 (Compulsory)	Saline for injection	iv	tail vein	daily	3	death

Appendix 4.3. Methodology information for in vitro MLD

MLD assays								
Lab	Source of Vero cells	Passage number	No. cells used for inoculation	Growth medium	% Fetal Calf Serum	Maintenance medium	% Fetal Calf Serum	Microtitre plate type
2	In House	238 -248	2 × 10^5/mL	M6B8	10	same as growth media	0	Falcon Tissue Culture Plate
3	In House	175	2.5-3 × 10^5	MEM5 (with EARLES F-15, 0.5 % LAH, 0.1 % Pen/Strep, 0.05 % Gent)	5	four times a day	0	Corning, Costar 3596
4	Laboratorio Nacional de Algete	2, 3 or 4 from cell bank	2 × 10^5/mL	DMEM	10	same as growth media	0	Costar 3599
5	White mice	142-158	40 000/well	MEM	10	same as growth media	0	BD Falcon 353072
6	ATCC	not reported	2 × 10^5/mL	MEM-H (HyClone) + 1 mg/L gentamycin	5	same as growth media	0	Nunc/Thermo, 161093
7	ATCC (CCL-81)	> 6	2 × 10^5/mL	DMEM with 10 mM HEPES; 2 mM L-glutamine; 30 µg/mL Gentamicin	5	same as growth media	0	Corning 3585 (sterile, flat-bottom, polystyrene, TC-treated)
8	ATCC	15-21	2 × 10^6/plate	MEM, Sigma	10	same as growth media	0	Costar 96 well
9	ATCC	4/6/ 10/ 14/ 16/ 1/ 9/ 11/ 13	10^4/well	GMEM	10	same as growth media	0	TPP 92096
10	VERO cells CRS batch 1 (ref. V0180000)	not reported	60 000/well	MEM	5	same as growth media	0	NUNC 96 flat bottom wells 353 072
11	Intervet (Vero L-251)	3	10^5/mL	MEM Earle's + 1 % NEA + 1 % Glutamine	10	same as growth media	0	Corning Costar 3596

Appendix 4.4. Methodology information for in vitro TCP

TCP assays								
Lab	Source of Vero cells	Passage number	No. cells used for inoculation	Growth medium	% FCS	Maintenance medium	% Fetal Calf Serum	Microtitre plate type
2	In house	265 - 280	2×10^5/mL	M6B8	10	same as growth media	0	Falcon Tissue Culture Plate
3	USDA NVSL Proficiency Testing and Reagents (Vero lot 9601)	146-155	$2.5\text{-}3 \times 10^5$	MEM5 (with EARLES F-15, 0.5 % LAH, 0.1 % Pen/Strep	not reported	same as growth media	0	Corning, Costar 3596
4	Laboratorio Nacional de Algete	4 - 6 from cell bank (preliminaries)	2×10^5/mL	DMEM	10	same as growth media	0	COSTAR 3599
5	ECACC VERO (WHO) Catalogue Number: 88020401	144-156	40 000/well	MEM	10	same as growth media	0	BD Falcon 353072
6	ATCC	22-24 from master cell bank	25 000/well	MEM-H (HyClone) + 1 mg/L gentamycin	5	same as growth media	0	Nunc/Thermo, 161093
7	ATCC (CCL-81)	5 , 8 , 9 , 10 , 6	2×10^5/mL	DMEM with 10 mM HEPES; 2 mM L-glutamine; 30 µg/mL Gentamicin	5	same as growth media	0	Corning 3585 (sterile, flat-bottom, poly-strene, TC-treated)
8	ATCC	15-25	2×10^6/plate	MEM Eagle, Sigma	10	same as growth media	0	Costar 96 well
9	ATCC	11/ 17/ 19/ 1/ 3	10^4/well	GMEM	10	same as growth media	0	TPP 92096
10	VERO cells CRS batch 1 (ref. V0180000)	4 -12 (Passage of the cell bank = 124)	60 000 to 70 000/well	MEM + 1 % L-Glu-tamine 200 mM + 1 % non essential amino acids + 1 % antibiot-ics (peni-strepto)	5	same as growth media	0	NUNC 96 flat bottom wells 353 072
11	Intervet (Vero L-251)	3	10^5/mL	MEM Earle's + 1 % NEA + 1 % Glutamine	10	same as growth media	0	Corning Costar 3596

Appendix 5. Statistical methods used in the central analysis

The method used in this report to calculate the Total Combining Power (TCP) of toxoids and the Toxin Equivalence (N) of toxins is the Maximum Likelihood (ML) method which consists in finding the model parameters that maximise the likelihood of the observed data as outlined below.

We start with 0.5 mL of antitoxin at a concentration of 4 IU/mL. The original tubes therefore contain 2 IU of antitoxin. Adding 0.5 mL of a toxoid with (unknown) binding power B expressed in IU/mL, diluted by a factor D can bind 0.5 × B/D of antitoxin. Since the amount of antitoxin cannot become negative this leaves

$$A = \mathrm{Max}\,(0\,;\,2 - 0.5 \times B/D)$$

antitoxin (in IU) in the tube. Adding 1.0 mL of detecting toxin with a (known or unknown) toxin equivalence N expressed in IU/mL, diluted by a factor L can bind a further 1.0 × N/L of antitoxin. Since the amount of detecting toxin cannot become negative this leaves

$$T_0 = \mathrm{Max}\,(0\,;\,1/L - A/N)$$

active detecting toxin in the tube, expressed in mL of pure substance. The total volume of the antitoxin/toxoid/toxin mix in the tube is 2 mL, of which 0.1 mL is transferred to the plate, possibly after applying a pre-dilution of a factor P. The content of pure unbound toxin in the 1st well is therefore

$$T_1 = 0.05 \times T_0/P$$

expressed in mL/well. The content in each subsequent well across the plate decreases by a factor 2 with each step. The content of the j-th well is therefore

$$T_j = T_1/2^{j-1}$$

expressed in mL/well of pure unbound toxin. All of the above equations can be put together in one big equation:

$$T_{i,j} = 0.05 \times \mathrm{Max}\,(0\,;\,1/L - \mathrm{Max}\,(0\,;\,2 - 0.5 \times B/D_i\,)/N)/P/2^{j-1}$$

where an extra index i for the other rows (tubes) on the plate is used.

Let S denote the (known or unknown) sensitivity of the Vero cells, expressed in mL/well of pure detecting toxin giving 50 % lethality. The tolerance distribution is given by

$$F(T) = f(a \times \ln(T/S))$$

where f is the logistic distribution function defined by

$$f(z) = 1/(1 + e^{-z})$$

The slope factor a can in theory be estimated from the data but to avoid over-parameterisation it has been somewhat arbitrarily set to a fixed value of $a = \ln(0.95/0.05)/\ln(2) \approx 4.25$ to force the probability level to raise from 5 % to 95 % over a 4-fold dilution. This value seems realistic because it is shallow enough to allow for occasional 2-fold shifts and steep enough to avoid frequent inversions.

Let $Y_{i,j}$ denote the actually observed responses expressed as 1 if positive (dead) and 0 if negative (life). The log-likelihood is then given by

$$(Y;b) = \sum_{i,j} Y_{i,j} \ln F\left(T_{i,j}\right) + (1 - Y_{i,j}) \ln(1 - F\left(T_{i,j}\right))$$

The parameter vector is symbolised by b and consists of the unknown parameters B, N and S. Having 3 unknown parameters in the model (or even 4 if the slope factor were to be estimated from the data as well) is problematic as it can easily give problems with convergence or yield estimates beyond reasonable boundaries. If good assumptions about the true values of N and/or S are available, as was the case in this study, they should be kept fixed so that only B enters the likelihood function as an unknown parameter. It is highly desirable that controls are included to monitor the correctness of these assumptions. If assumptions are not available

it becomes almost a necessity to include additional information into the model such as data from the toxin/antitoxin test (VI test) and the toxin sensitivity tests. The VI test would enter the equation as

$$T_{i,j} = 0.05 \times \text{Max}\,(0\,;\,1/L - U_i/N)/P/2^{j-1}$$

Where Ui is the amount of antitoxin expressed in IU/tube. This can be easily derived from the TCP equation by setting $B = 0$ and replacing the constant 2 by U_i The equation for the toxin sensitivity test would simply be

$$T_j = 0.1/P/2^{j-1}$$

Note that P may be different in each type of assay. All of the above equations might be used in one compound optimisation for all replicate plates and types of tests to obtain one simultaneous estimate for B, N, S and possibly even for a.

The algorithm used to find the maximum likelihood parameters is the downhill simplex method due to Nelder and Mead [1]. This method was chosen because of its robust properties for non-differentiable (but continuous) objective functions, as is the case in this study. This method is available as 'optim( )' in the core package of the free software R. Unknown parameters were initialised at $B = 100$ IU/mL, $N = 284$ IU/mL and $S = 0.5$ nL/well. Example scripts are provided in Appendix 7.

[1] Nelder, J.A., and Mead, R. 1965 Computer Journal, vol. 7, pp. 308-313.

Appendix 6. Examples of determination of endpoints in *in vitro* TCP experiments

We consider here an example to clarify the problem of equal endpoint on all rows of the TCP assay. Let us assume a toxin equivalence of $N = 284$ IU/mL and a sensitivity of $S = 0.5$ nL/well. A toxoid such as TdK can have a binding power as high as $B = 180$ IU/mL. If 5 tubes are prepared at 140, 160, 180, 200, 220 TCP units with $L^+ = 1/170$ mL the remaining amount of detecting toxin in the 2 mL tube is 3367, 2801, 2361, 2009, 1721 nL respectively (see diagram hereunder).

TCP units	0.5mL antitoxin	+	0.5mL toxoid	→	Antitoxin in 1mL mix	+	1mL toxin	→	Toxin in 2mL mix
1/70 =140 TCP units	2IU		7143nL		0.714IU		5882nL		3367nL
1/80 =160 TCP units	2IU		6250nL		0.875IU		5882nL		2801nL
1/90 =180 TCP units	2IU		5556nL		1.000IU		5882nL		2361nL
1/100 =200 TCP units	2IU		5000nL		1.100IU		5882nL		2009nL
1/110 =220 TCP units	2IU		4545nL		1.182IU		5882nL		1721nL

Already at this stage it is clear that the remaining toxin in the final mix differs by less than a factor 2 between the first and last tube so one can expect at most 1 well difference in the end-points. Depending on the exact sensitivity of the Vero cells this one-well difference may occur on any row and can therefore not directly be correlated to dead/life responses in mice. Indeed, expected responses when plated at a pre-dilution of 1/16 are as shown below:

Sensitivity = 0.5nL/well

A												
B		10.5	5.26	2.63	1.32	0.66	0.33	0.16	0.08	0.04	0.02	
C		8.8	4.38	2.19	1.09	0.55	0.27	0.14	0.07	0.03	0.02	
D		7.4	3.69	1.84	0.92	0.46	0.23	0.12	0.06	0.03	0.01	
E		6.3	3.14	1.57	0.78	0.39	0.20	0.10	0.05	0.02	0.01	
F		5.4	2.69	1.34	0.67	0.34	0.17	0.08	0.04	0.02	0.01	
G												
H												

Sensitivity=0.34nL/well

A												
B		10.5	5.26	2.63	1.32	0.66	0.33	0.16	0.08	0.04	0.02	
C		8.8	4.38	2.19	1.09	0.55	0.27	0.14	0.07	0.03	0.02	
D		7.4	3.69	1.84	0.92	0.46	0.23	0.12	0.06	0.03	0.01	
E		6.3	3.14	1.57	0.78	0.39	0.20	0.10	0.05	0.02	0.01	
F		5.4	2.69	1.34	0.67	0.34	0.17	0.08	0.04	0.02	0.01	
G												
H												

Values are the toxin contents in nL/well. Shaded wells indicate expected death and light cells indicate expected survival based on a true underlying sensitivity of 0.5 nL/well. If the Vero cells have a sensitivity of 0.34 nL/well, one could easily find that all rows give the same endpoint. This demonstrates the impossibility of finding a satisfactory 1-on-1 correlation between end-points on Vero cells and mortality in mice with the chosen design. The ML-method applied to these examples, assuming N = 284 IU/mL and S = 0.5 nL/well yields B = 180 IU/mL for the left plate and 244 IU/mL for the right plate, which demonstrates how the outcome depends on assumptions about sensitivity. Worse even, if no assumptions about S and N were available and also had to be estimated from the observed data, the outcome becomes even more unstable as can be seen in the following table. In the next tables, values marked with a star are kept fixed whereas values without a star are estimated from the observed data

True sensitivity = 0.5 nL/well		
B (IU/mL)	N (IU/mL)	S (nL/well)
180	284*	0.500*
134	284*	0.308
197	258	0.500*
199	257	0.505

True sensitivity = 0.34 nL/well		
B (IU/mL)	N (IU/mL)	S (nL/well)
244	284*	0.500*
507	284*	0.812
1	881	0.500*
49	7161	0.779

There are several ways the design could be changed to improve the situation. A theoretical solution would be to use higher dilutions (lower concentrations) of the detecting toxin so that the levels of remaining toxin after incubation are closer to 0 and therefore more easily show n-fold differences. For example with L^+ = 1/240 mL and without pre-dilution before plating the responses are expected to be like this:

Sensitivity = 0.5nL/well

A												
B		82.6	41.3	20.6	10.3	5.16	2.58	1.29	0.65	0.32	0.16	
C		54.3	27.1	13.6	6.79	3.39	1.70	0.85	0.42	0.21	0.11	
D		32.3	16.1	8.07	4.03	2.02	1.01	0.50	0.25	0.13	0.06	
E		14.7	7.34	3.67	1.83	0.92	0.46	0.23	0.11	0.06	0.03	
F		0.27	0.13	0.07	0.03	0.02	0.01	0.00	0.00	0.00	0.00	
G												
H												

Sensitivity=0.34nL/well

A												
B		82.6	41.3	20.6	10.3	5.16	2.58	1.29	0.65	0.32	0.16	
C		54.3	27.1	13.6	6.79	3.39	1.70	0.85	0.42	0.21	0.11	
D		32.3	16.1	8.07	4.03	2.02	1.01	0.50	0.25	0.13	0.06	
E		14.7	7.34	3.67	1.83	0.92	0.46	0.23	0.11	0.06	0.03	
F		0.27	0.13	0.07	0.03	0.02	0.01	0.00	0.00	0.00	0.00	
G												
H												

The ML-method applied to these examples, assuming N = 284 IU/mL and S = 0.5 nL/well yields B = 179 IU/mL for the left plate and B = 180 IU/mL for the right plate, showing that the result is fairly robust against small departures from the assumed sensitivity. Below is a summary table with results using several combinations of fixed and free parameters.

True sensitivity = 0.5 nL/well		
B (IU/mL)	N (IU/mL)	S (nL/well)
179	284*	0.500*
175	284*	0.417
194	263	0.500*
192	264	0.492

True sensitivity = 0.34 nL/well		
B (IU/mL)	N (IU/mL)	S (nL/well)
180	284*	0.500*
180	284*	0.351
218	242	0.500*
330	112	1.214

The disadvantage of this design is that the dilution of the toxin expects prior knowledge about the binding power of the toxoid. For toxoids with a lower binding power an L^+ of 1/240 mL could lead to complete neutralisation of the detecting toxin in all tubes leaving no information at all on the binding power of the toxoid.

Another option is to use larger steps between toxoid dilutions. The current design uses equal steps of 20 TCP units but one could envisage a geometric progression such as 20, 40, 80, 160, 320 TCP units. Assuming that all underlying parameters are the same as above (L^+ = 1/170 mL, N = 284 IU/mL, B = 180 IU/mL) this would give the following expected responses at a pre-dilution of 1/16:

Sensitivity = 0.5nL/well

A											
B		18.4	9.19	4.60	2.30	1.15	0.57	0.29	0.14	0.07	0.04
C		18.4	9.19	4.60	2.30	1.15	0.57	0.29	0.14	0.07	0.04
D		18.4	9.19	4.60	2.30	1.15	0.57	0.29	0.14	0.07	0.04
E		8.75	4.38	2.19	1.09	0.55	0.27	0.14	0.07	0.03	0.02
F		2.56	1.28	0.64	0.32	0.16	0.08	0.04	0.02	0.01	0.01
G											
H											

Sensitivity=0.34nL/well

A											
B		18.4	9.19	4.60	2.30	1.15	0.57	0.29	0.14	0.07	0.04
C		18.4	9.19	4.60	2.30	1.15	0.57	0.29	0.14	0.07	0.04
D		18.4	9.19	4.60	2.30	1.15	0.57	0.29	0.14	0.07	0.04
E		8.75	4.38	2.19	1.09	0.55	0.27	0.14	0.07	0.03	0.02
F		2.56	1.28	0.64	0.32	0.16	0.08	0.04	0.02	0.01	0.01
G											
H											

The ML-method using various combinations of fixed and free parameters gives:

True sensitivity = 0.5 nL/well		
B (IU/mL)	N (IU/mL)	S (nL/well)
195	284*	0.500*
175	284*	0.401
226	260	0.500*
192	272	0.406

True sensitivity = 0.34 nL/well		
B (IU/mL)	N (IU/mL)	S (nL/well)
195	284*	0.500*
175	284*	0.401
226	260	0.500*
192	272	0.406

The advantage of this design is that it can be used for a wide range of toxoids and is therefore certainly suitable as a preliminary ranging test. It also appears to be reasonably accurate, even without assumptions about N and S. Below is another example with the same design but for a low toxoid similar to TdJ with B = 30 IU/mL.

Sensitivity = 0.5nL/well

A											
B		12.9	6.44	3.22	1.61	0.81	0.40	0.20	0.10	0.05	0.03
C		4.63	2.31	1.16	0.58	0.29	0.14	0.07	0.04	0.02	0.01
D		0.50	0.25	0.13	0.06	0.03	0.02	0.01	0.00	0.00	0.00
E		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
F		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
G											
H											

Sensitivity=0.34nL/well

A											
B		12.9	6.44	3.22	1.61	0.81	0.40	0.20	0.10	0.05	0.03
C		4.63	2.31	1.16	0.58	0.29	0.14	0.07	0.04	0.02	0.01
D		0.50	0.25	0.13	0.06	0.03	0.02	0.01	0.00	0.00	0.00
E		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
F		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
G											
H											

True sensitivity = 0.5 nL/well		
B (IU/mL)	N (IU/mL)	S (nL/well)
31	284*	0.500*
32	284*	0.531
29	289	0.500*
51	247	0.812

True sensitivity = 0.34 nL/well		
B (IU/mL)	N (IU/mL)	S (nL/well)
32	284*	0.500*
30	284*	0.334
40	265	0.500*
39	265	0.475

Yet another alternative would be to target the complete neutralisation between the 3rd or 4th row. The 1st and 5th rows would serve as a positive and negative control respectively. The endpoints will change sharply near the middle row, providing a very accurate estimate of the remaining detecting toxin and hence, the binding power of the toxoid. An L+ of 190 (= N/1.5), which leaves about 0.5 IU of active detecting toxin on the middle row could be used for this purpose. The sharp change of endpoint may also be easier to interpret as predictions for the survival rates in mice.

There are other designs that could be envisaged, such as fixed toxoid dilutions but varying toxin dilutions, or fixed toxoid and toxin dilutions but varying antitoxin dilutions. Lower quantities of antitoxin, toxin and toxoid may save material. The advantages and disadvantages of these approaches will need further consideration, both from the practical point of view as from the computational point of view by doing more elaborate simulations than could be presented in this Appendix.

We conclude with an example of an assay design that could be used to express toxicity of toxins in IU/mL (the toxin equivalence) instead of the MLD. The proposed design is very similar

to the toxin/antitoxin test used for the CSTx in this study but in order to save material, lower quantities of the components are proposed and the antitoxin is used at fixed levels whereas the toxin concentration decreases on each next row.

In brief: Prepare 2 series of 6 tubes with 1 mL of test toxin at dilutions of 1/5, 1/25, 1/125, 1/625, 1/3125, 1/15625 (5-fold series starting with 1/5). To one series add 1 mL of 0.1 IU/mL antitoxin. To the other series add 1 mL of buffer solution (no antitoxin). Load 0.1 mL from the first series onto a microtitre plate and 0.1 mL from the second series onto another microtitre plate. Then make 2-fold dilutions across the plates.

This design allows for direct estimation of the MLD on one plate and the toxin equivalence from the combination of both plates because the effect of the antitoxin can be directly related to the known toxin concentrations on the plate. It is suitable for values in the range from 1 to 1000 IU/mL. Here is an example of the expected read-outs, assuming a true sensitivity of 150 µIU/well and a true toxin equivalence of 80 IU/mL.

Without antitoxin

A											
B		>>	>>	>>	>>	>>	>>	>>	>>	>>	>>	
C		>>	>>	>>	>>	>>	>>	>>	>>	625	313	
D		>>	>>	>>	>>	>>	>>	500	250	125	62.5	
E		>>	>>	>>	800	400	200	100	50	25	12.5	
F		>>	>>	>>	160	80	40	20	10	5	2.5	
G		256	128	64	32	16	8	4	2	1	0.5	
H												

With antitoxin

A											
B		>>	>>	>>	>>	>>	>>	>>	>>	>>	>>	
C		>>	>>	>>	>>	>>	>>	>>	>>	605	303	
D		>>	>>	>>	>>	>>	844	422	211	105	52.7	
E		>>	700	350	175	88	44	22	11	5.5	2.7	
F		0	0	0	0	0	0	0	0	0	0	
G		0	0	0	0	0	0	0	0	0	0	
H												

The ML-method applied to these read-outs yields 84.74 IU/mL as an estimate of the toxin equivalence and a sensitivity of 157.3 µIU/well. Both estimates are quite close to the true values but it is possible to perform a new assay with toxin dilutions more closely bracketed around 1/850 (= 85 × 10) to achieve higher precision. Other designs can of course also be envisaged. The above example is only intended to illustrate the concept of toxin equivalence.

Appendix 7. Calculation methodology using the free software package R

The maximum likelihood method used in this study was implemented in the free software package R by use of the built-in function ‘optim’. This function expects an initial guess of the parameters to be optimised, an objective function which expresses the log-likelihood of the observed data for a given set of parameters, and the assay data. The objective function requires the assay data to be a dataframe with one line for each valid observation (one line per well) and the following numeric variables:

```
Ucon = the concentration of antitoxin in IU/mL (typically 4 in TCP assays)

Uvol = the volume of antitoxin in mL (typically 0.5 in TCP assays)

Bdil = the dilution factor of the toxoid (e.g. 25 for the case 50 TCP units)

Bvol = the volume of toxoid in mL (typically 0.5)

Ldil = the dilution factor of the detecting toxin (in this study typically 170)

Lvol = the volume of detecting toxin in mL (typically 1.0)

Wpre = the predilution factor applied before plating the first well (e.g. 16)

Wvol = the volume applied to the wells in mL (typically 0.1)

Wstp = the dilution step between wells (typically 2)

Wnbr = the index of the well number (in the range 1 to 10)

Yobs = the observed response (1=dead, 0=life)
```

This data format allows for very flexible data input where each individual well can be controlled independently. It can be used to contain data from TCP assays, MLD assays and VI assays. Because in practice most assays have a simple design, several convenience functions are also provided here for easy generation of the required dataset. Rows with irregular sequences of positive and negative wells should not be included.

```
TCPassay<-function(D,L,P,Y){

## D=vector of toxoid dilutions, L=dilution factor of toxin, P=predilution,

## Y=vector of endpoints (number of dead wells out of 10).

  Ucon<-4; Uvol<-0.5; Bdil<-D; Bvol<-0.5; Ldil<-L; Lvol<-1

  Wpre<-P; Wvol<-0.1; Wstep<-2; Wnbr<-rep(1:10,each=length(Y))

  Yobs<-as.integer(Wnbr<=rep(Y,10))

  data.frame(Ucon,Uvol,Bdil,Bvol,Ldil,Lvol,Wpre,Wvol,Wstep,Wnbr,Yobs)

}


VIassay<-function(U,L,P,Y){

## U=vector of antitoxin concentrations, L=dilution factor of toxin,

## P=predilution, Y=vector of endpoints (number of dead wells out of 10).

  Ucon<-U; Uvol<-1; Bdil<-1; Bvol<-0; Ldil<-L; Lvol<-1

  Wpre<-P; Wvol<-0.1; Wstep<-2; Wnbr<-rep(1:10,each=length(Y))

  Yobs<-as.integer(Wnbr<=rep(Y,10))

  data.frame(Ucon,Uvol,Bdil,Bvol,Ldil,Lvol,Wpre,Wvol,Wstep,Wnbr,Yobs)

}


MLDassay<-function(L,P,Y){

## L=vector of toxin dilutions, P=predilution, Y=vector of endpoints.

  Ucon<-1; Uvol<-0; Bdil<-1; Bvol<-0; Ldil<-L; Lvol<-1

  Wpre<-P; Wvol<-0.1; Wstep<-2; Wnbr<-rep(1:10,each=length(Y))

  Yobs<-as.integer(Wnbr<=rep(Y,10))

  data.frame(Ucon,Uvol,Bdil,Bvol,Ldil,Lvol,Wpre,Wvol,Wstep,Wnbr,Yobs)

}
```

The objective function is as described in Appendix 5. It requires as input the dataset generated above and values for the 4 parameters B, N, S and a. It returns the log-likelihood.

```
fL<-function(assay,B,N,S,a){

  with(assay,{

   A<-pmax(0,Ucon*Uvol-Bvol*B/Bdil)

   T0<-pmax(0,1/Ldil-A/N)

   T1<-Wvol/(Uvol+Bvol+Lvol)*T0/Wpre

   T<-1000000*T1/(2^(Wnbr-1))
```

```
   z<-a*log(T/S)
   F<-1/(1+exp(-z))
   sum(log(Yobs*F+(1-Yobs)*(1-F)))
  })
}
```

The function fOptim is a wrapper for the built-in function optim. It handles some overhead to separate the free parameters from the fixed parameters, and initialises parameters at reasonable values if not provided by the calling function. The parameters to be optimised are passed as a string, e.g. 'BS' will optimise the binding power and the sensitivity but will keep the toxin equivalence and slope fixed at their initial values (defaults are used if not provided by the calling function).

```
fOptim<-function(assay,free='BNSa',B=100,N=284,S=0.5,a=log(0.95/0.05)/log(2)){
  p<-setNames(c(B,N,S,a),c('B','N','S','a'))
  free<-strsplit(free,'')[[1]]
  pfree<-p[free]
  fix<-'BNSa'
  for (i in free) {fix<-gsub(i,'',fix)}
  fix<-strsplit(fix,'')[[1]]
  pfix<-p[fix]
  f<-function(pfree,pfix,assay){
   p<-c(exp(pfree),exp(pfix))
   -fL(assay,p['B'],p['N'],p['S'],p['a'])
  }
  for(i in 1:10){
   result<-suppressWarnings(optim(log(pfree),f,pfix=log(pfix),assay=assay))
   pfree<-exp(result$par)
  }
  result$par<-exp(result$par)
  result
}
```

Example call for the CSTx sensitivity test:

```
Dil<-c(1,3,9,27,81,243)
ThisAssay<-MLDassay(Dil,1000,c(10,9,7,6,4,2,10,8,7,6,4,2))
fOptim(ThisAssay,'S')
## Output S=0.114 which means that the sensitivity of the Verocells is 0.114nL CSTx/well. The MLD is
a factor sqrt(2) higher than this value.
```

Example call for a test toxin:

```
Dil<-c(1,3,9,27,81)

ThisAssay<-MLDassay(Dil,100,c(10,8,6,5,3,10,8,6,5,3))

fOptim(ThisAssay,'S')

## Output S=1.843 which means that the LD50 of this toxin is estimated as 1.843nL/well. The MLD is a
factor sqrt(2) higher than this value.
```

Example calls for the VI test:

```
Dil<-c(1.50,1.25,1.00,0.75,0.50)

ThisAssay<-VIassay(Dil,170,1,c(7,8,8,9,10,7,8,9,9,10))

fOptim(ThisAssay,'S')

## Output S=0.375. The sensitivity assuming N=284 is estimated as 0.375nL/well.

fOptim(ThisAssay,'NS')

## Output N=287, S=0.388. The sensitivity without assumptions about N is

## estimated as 0.388nL/well.

fOptim(ThisAssay,'N',S=0.400)

## Output N=289. The toxin equivalence is estimated as 289IU/mL, assuming a

## sensitivity of 0.400nL/well.
```

Example calls for the TCP test:

```
Dil<-c(20,30,40,50,60)

ThisAssay<-TCPassay(Dil,170,16,c(6,6,6,6,6,7,7,7,7,6))

fOptim(ThisAssay,'B',S=0.262)

## Output B=177. The binding power is estimated as 177IU/mL, assuming a

## sensitivity of 0.262nL/well.

fOptim(ThisAssay,'BS')

## Output B=190, S=0.287. The binding power without assumptions on

## sensitivity (but assuming N=284) is estimated as 190IU/mL.

fOptim(ThisAssay,'BNS')

## Ouput B=201, N=188, S=0.287. The binding power without making any

## assumptions on N and S is estimated as 201IU/mL.
```

Table A – MLD in vivo full testing. Summary overview of endpoints

	TxA			TxB			TxC			TxD			TxE			TxF		
	Assay 1	Assay 2	Assay 3	Assay 1	Assay 2	Assay 3	Assay 1	Assay 2	Assay 3	Assay 1	Assay 2	Assay 3	Assay 1	Assay 2	Assay 3	Assay 1	Assay 2	Assay 3
Pre-dilution	27	27	27	16.67	16.67	16.67	3	3	3	3	3	3	50	50	50	50	50	50
Lab 1	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D
	3 D;D	3 D;D	3 D;D	3 D;D	3 D;D	3 D;D	3 D;D	3 L;L	3 L;L	3 D;D	3 D;D	3 D;D	3 D;D	3 D;D	3 D;D	3 D;D	3 D;D	3 D;D
	9 L;L	9 L;L	9 L;L	9 L;L	9 L;L	9 L;L	9 L;L	9 L;L	9 L;L	9 L;L	9 L;L	9 L;L	9 L;L	9 L;L	9 L;L	9 L;L	9 L;L	9 L;L
	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L
	81 L;L	81 L;L	81 –	81 L;L	81 L;L	81 –	81 L;L	81 L;L	81 –	81 L;L	81 L;L	81 –	81 L;L	81 L;L	81 –	81 L;L	81 L;L	81 –
Pre-dilution	50	50	50	50	50	50	3	3	3	10	10	10	50	50	50	50	50	50
Lab 2	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D
	3 L;L	3 D;D	3 D;D	3 D;D	3 D;D	3 D;D	3 D;D	3 D;L	3 D;D	3 D;D	3 D;L	3 L;L	3 D;D	3 D;D	3 D;D	3 D;D	3 D;L	3 D;D
	9 L;L	9 L;L	9 L;L	9 L;L	9 L;L	9 L;L	9 L;L	9 L;L	9 L;L	9 L;L	9 L;L	9 L;L	9 L;L	9 L;L	9 L;L	9 L;L	9 L;L	9 L;L
	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L
	81 L;L	81 L;L	81 L;L	81 L;L	81 L;L	81 L;L	81 L;L	81 L;L	81 L;L	81 L;L	81 L;L	81 L;L	81 L;L	81 L;L	81 L;L	81 L;L	81 L;L	81 L;L
Pre-dilution	50	100	100	100	100	200	8	6	8	8	10	10	100	100	200	100	100	200
Lab 3	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D	1 D	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D
	3 D;D	3 D;D	3 D;L	3 D;D	3 D;D	3 L;L	3 D;L	3 D;D	3 D;L	3 D;D	3 D;D	3 D;D	3 D;D	3 D;D	3 L;L	3 D;D	3 D;D	3 L;L
	9 D;D	9 L;L	9 L;L	9 L;L	9 L;L	9 L;L	9 L;L	9 L;L	9 L;L	9 L;L	9 L;L	9 L;L	9 L;L	9 D;L	9 L;L	9 L;L	9 L;L	9 L;L
	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L
	81 L;L	81 L;L	81 L;L	81 L;L	81 L;L	81 L;L	81 L;L	81 L;L	81 L;L	81 L;L	81 L;L	81 L;L	81 L;L	81 L;L	81 L;L	81 L;L	81 L;L	81 L;L

Lab 3 performed a 4th assay with TxC and TxD because Assay 1 was invalid on cells. Assays 2 to 4 are listed above. Assay 1 gave D;D-L;L-L;L-L;L-L;L for TxC and D;D-D;D-L;L-L;L-L;L for TxD (pre-dilution 1/10).

	TxA			TxB			TxC			TxD			TxE			TxF		
	Assay 1	Assay 2	Assay 3	Assay 1	Assay 2	Assay 3	Assay 1	Assay 2	Assay 3	Assay 1	Assay 2	Assay 3	Assay 1	Assay 2	Assay 3	Assay 1	Assay 2	Assay 3
Pre-dilution	4	4	4	4	4	4	1	1	1	1	1	1	4	4	4	4	4	4
Lab 4	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D	1 –	1	1	1	3 D;D	3 D;D	3 D;D	3 D;D	3 D;D	3 D;D
	3 D;D	3 D;D	3 D;D	3 D;D	3 D;D	3 D;D	3 D;D	3 D;D	3 –	3 D;D	3 D;D	3 D;D	9 D;D	9 D;D	9 D;D	9 D;D	9 D;D	9 D;D
	9 D;D	9 D;D	9 D;D	9 L;L	9 D;D	9 D;D	9 L;L	9 L;L	9 –	9 D;L	9 D;D	9 L;L	27 L;L	27 L;L	27 D;D	27 L;L	27 L;L	27 L;L
	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L	27 –	27 L;L	27 L;L	27 L;L	81 L;L	81 L;L	81 L;L	81 L;L	81 L;L	81 L;L
	81 L;L	81 L;L	81 L;L	81 L;L	81 L;L	81 L;L	81 L;L	81 L;L	81 –	81 L;L	81 L;L	81 L;L	243 L;L	243 L;L	243 L;L	243 L;L	243 L;L	243 L;L
Pre-dilution	10	10	10	5	5	5	1	1	1	3	3	3	5	5	5	5	5	5
Lab 5	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D	1 –	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D
	3 L;L	3 D;D	3 D;D	3 D;D	3 D;D	3 D;D	3 D;D	3 L;L	3 –	3 D;D	3 L;L	3 D;D	3 D;D	3 D;D	3 D;D	3 D;D	3 D;D	3 D;D
	9 L;L	9 L;L	9 L;L	9 D;D	9 L;L	9 D;D	9 L;L	9 L;L	9 –	9 L;L	9 L;L	9 L;L	9 D;D	9 D;L	9 D;D	9 D;D	9 D;D	9 D;D
	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L	27 –	27 L;L	27 L;L	27 L;L	27 D;D	27 L;L	27 D;D	27 D;L	27 L;L	27 D;D
	81 L;L	81 L;L	81 L;L	81 L;L	81 L;L	81 L;L	81 L;L	81 L;L	81 –	81 L;L	81 L;L	81 L;L	81 L;L	81 L;L	81 L;L	81 L;L	81 L;L	81 L;L
Pre-dilution	28	28	28	17	17	17	3.3	3.3	3.3	2	2	2	17	17	17	17	17	17
Lab 6	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D	1 D;D
	3 D;D	3 D;D	3 D;D	3 D;D	3 D;D	3 D;D	3 D;L	3 D;D	3 D;D	3 D;D	3 D;D	3 D;D	3 D;D	3 D;D	3 D;D	3 D;D	3 D;D	3 D;D
	9 L;L	9 L;L	9 L;L	9 L;L	9 D;D	9 D;D	9 L;L	9 L;L	9 L;L	9 D;L	9 D;D	9 D;D	9 D;D	9 D;D	9 D;D	9 D;L	9 D;D	9 D;D
	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L	27 L;L
	81 L;L	81 L;L	81 L;L	81 L;L	81 L;L	81 L;L	81 –	81 –	81 –	81 –	81 –	81 –	81 L;L	81 L;L	81 L;L	81 L;L	81 L;L	81 L;L

Table B – MLD in vitro full testing. Summary overview of endpoints

	TxA						TxB						TxC						TxD						TxE						TxF					
	Assay 1		Assay 2		Assay 3		Assay 1		Assay 2		Assay 3		Assay 1		Assay 2		Assay 3		Assay 1		Assay 2		Assay 3		Assay 1		Assay 2		Assay 3		Assay 1		Assay 2		Assay 3	
Pre-dilution	50		50		50		50		50		50		3		3		3		10		10		10		50		50		50		50		50		50	
Lab 2 Ref = 1:2500	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D	1	9;9	1	9;D	1	D;D	1	9;9	1	9;8	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D
	3	8;8	3	8;8	3	9;9	3	9;9	3	9;9	3	8;8	3	8;8	3	7;7	3	8;8	3	9;9	3	7;7	3	8;7	3	D;D	3	8;9	3	9;9	3	D;D	3	8;8	3	9;9
	9	7;7	9	6;6	9	8;8	9	8;8	9	7;7	9	7;7	9	7;6	9	5;5	9	7;7	9	7;7	9	6;6	9	5;5	9	8;8	9	6;7	9	7;7	9	8;8	9	6;6	9	7;7
	27	5;5	27	5;5	27	7;6	27	6;6	27	6;6	27	6;6	27	5;5	27	4;4	27	6;6	27	5;6	27	5;5	27	4;4	27	6;6	27	5;6	27	6;6	27	6;6	27	5;5	27	6;5
	81	3;3	81	3;3	81	5;5	81	5;4	81	4;4	81	4;4	81	4;4	81	3;3	81	4;4	81	4;4	81	4;4	81	3;3	81	5;4	81	3;4	81	4;5	81	4;5	81	4;4	81	5;4
	Ref	8;8	Ref	8;9	Ref	8;8	Ref	8;8	Ref	8;8	Ref	8;7	Ref	7;8	Ref	7;8	Ref	8;8	Ref	8;8	Ref	8;8	Ref	7;8	Ref	8;7	Ref	7;7	Ref	8;8	Ref	8;8	Ref	7;8	Ref	8;7
Pre-dilution	50		100		100		100		100		200		8		6		8		8		10		10		100		100		200		100		100		200	
Lab 3 Ref = 1:40000	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D	1	9;9	1	D;D	1	D;D	1	D;9	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D	1	9;D	1	D;D	1	D;D	1	D;D
	3	9;9	3	9;9	3	8;8	3	8;9	3	8;8	3	7;7	3	8;8	3	8;8	3	8;8	3	9;9	3	D;D	3	8;8	3	8;8	3	D;D	3	7;8	3	8;8	3	9;9	3	8;8
	9	7;7	9	7;7	9	6;6	9	6;6	9	6;6	9	5;5	9	6;6	9	6;6	9	6;6	9	7;8	9	8;8	9	6;6	9	5;6	9	8;8	9	6;6	9	6;6	9	8;7	9	6;6
	27	6;6	27	5;5	27	4;4	27	5;5	27	5;5	27	X;X	27	5;5	27	4;4	27	4;4	27	5;6	27	6;6	27	4;4	27	4;5	27	6;6	27	4;4	27	5;5	27	6;6	27	4;4
	81	4;4	81	4;4	81	2;3	81	3;X	81	3;3	81	X;2	81	3;3	81	3;3	81	3;3	81	4;4	81	5;5	81	3;3	81	2;3	81	4;4	81	2;3	81	3;3	81	4;4	81	3;3
	Ref	4;3	Ref	5;5	Ref	4;4	Ref	4;4	Ref	4;4	Ref	X;4	Ref	5;5	Ref	5;4	Ref	5;4	Ref	5;5	Ref	5;5	Ref	4;4	Ref	4;4	Ref	5;5	Ref	5;5	Ref	4;4	Ref	5;5	Ref	5;5
Pre-dilution	64		64		64		64		64		64		16		16		16		16		16		16		64		64		64		64		64		64	
Lab 4 Ref = 1:12000	1	8;8	1	8;8	1	8;8	1	7;7	1	7;7	1	9;9	1	6;6	1	5;5	1	6;6	1	8;8	1	8;9	1	9;9	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D
	3	6;6	3	6;6	3	7;6	3	6;6	3	5;5	3	7;7	3	5;5	3	4;3	3	5;5	3	7;7	3	7;7	3	7;7	3	8;8	3	9;9	3	9;9	3	9;8	3	9;9	3	8;9
	9	4;5	9	5;5	9	5;5	9	4;4	9	4;4	9	5;5	9	3;3	9	2;2	9	3;3	9	5;5	9	6;6	9	5;5	9	6;7	9	7;7	9	7;7	9	7;6	9	7;7	9	7;7
	27	3;3	27	3;3	27	4;4	27	3;3	27	2;2	27	4;4	27	1;2	27	L;L	27	1;2	27	3;3	27	4;4	27	3;3	27	5;5	27	5;5	27	5;5	27	5;5	27	5;5	27	5;5
	81	2;2	81	2;2	81	2;2	81	1;1	81	1;1	81	2;2	81	L;L	81	L;L	81	L;L	81	1;2	81	2;2	81	1;2	81	3;3	81	4;3	81	3;3	81	3;3	81	3;3	81	3;3
	Ref	5;5	Ref	X;5	Ref	6;6	Ref	5;4	Ref	5;4	Ref	5;6	Ref	5;5	Ref	4;4	Ref	5;5	Ref	5;5	Ref	5;5	Ref	5;5	Ref	4;5	Ref	5;5	Ref	5;5	Ref	4;4	Ref	5;5	Ref	5;5

Lab 4 performed a 4th assay with TxC and TxD. The results are TxC: 8;8 - 6;6 - 4;4 - 3;3 - 1;1 (Ref 5;5)/TxD: 8;8 - 6;6 - 5;5 - 3;3 - 1;1 (Ref 4;5).

	TxA			TxB			TxC			TxD			TxE			TxF		
	Assay 1	Assay 2	Assay 3	Assay 1	Assay 2	Assay 3	Assay 1	Assay 2	Assay 3	Assay 1	Assay 2	Assay 3	Assay 1	Assay 2	Assay 3	Assay 1	Assay 2	Assay 3
Pre-dilution	64	64	64	64	64	64	16	16	16	16	16	16	64	64	64	64	64	64
Lab 5 Ref = 1:12150	1 7;6	1 6;6	1 6;6	1 6;6	1 6;6	1 6;6	1 6;7	1 7;6	1 7;8	1 D;D	1 D;D	1 D;D	1 8;8	1 8;8	1 8;8	1 8;8	1 9;9	1 9;9
	3 5;5	3 5;5	3 5;5	3 5;4	3 5;5	3 5;5	3 5;5	3 5;5	3 6;6	3 9;9	3 9;9	3 9;9	3 6;6	3 6;6	3 6;6	3 7;7	3 8;8	3 8;7
	9 4;4	9 4;4	9 4;3	9 3;3	9 3;3	9 3;3	9 4;4	9 4;3	9 4;4	9 7;7	9 7;8	9 8;8	9 5;5	9 5;5	9 5;5	9 5;5	9 6;6	9 6;6
	27 2;2	27 2;2	27 2;2	27 2;1	27 2;2	27 2;2	27 2;2	27 2;2	27 3;3	27 6;6	27 6;6	27 6;6	27 3;3	27 3;3	27 4;4	27 4;4	27 5;5	27 4;4
	81 1;1	81 1;1	81 1;1	81 L;L	81 L;L	81 1;L	81 L;L	81 1;L	81 2;2	81 4;5	81 4;4	81 5;5	81 2;2	81 2;2	81 2;2	81 2;2	81 3;3	81 3;3
	Ref 5;5	Ref 6;6	Ref 6;6	Ref 5;5	Ref 5;5	Ref 6;6	Ref 5;5	Ref 6;6	Ref 6;6	Ref 6;6	Ref 6;6	Ref 6;6	Ref 4;4	Ref 6;6	Ref 6;6	Ref 5;5	Ref 6;6	Ref 6;6
Pre-dilution	224	224	224	136	136	136	26.4	26.4	26.4	16	16	16	136	136	136	136	136	136
Lab 6 Ref = 1:40000	1 9;9	1 7;7	1 7;7	1 8;8	1 7;7	1 7;7	1 7;7	1 5;5	1 5;5	1 D;D	1 D;D	1 9;9	1 D;D	1 9;8	1 8;8	1 D;D	1 9;9	1 9;9
	3 7;7	3 5;5	3 5;5	3 7;6	3 5;6	3 5;6	3 5;5	3 X;4	3 3;3	3 8;8	3 8;8	3 8;8	3 8;8	3 7;6	3 7;6	3 8;8	3 8;7	3 8;7
	9 6;6	9 4;4	9 4;4	9 5;4	9 4;4	9 4;4	9 4;4	9 2;2	9 2;2	9 7;7	9 7;7	9 6;6	9 6;6	9 6;5	9 5;5	9 6;6	9 6;6	9 6;6
	27 4;4	27 2;2	27 2;2	27 3;3	27 3;3	27 3;3	27 2;3	27 1;1	27 L;L	27 5;5	27 5;5	27 5;4	27 5;5	27 4;4	27 4;4	27 5;5	27 4;4	27 5;4
	81 2;3	81 1;1	81 1;1	81 2;2	81 1;1	81 1;1	81 1;1	81 L;L	81 L;L	81 4;3	81 3;3	81 3;3	81 4;4	81 3;2	81 2;2	81 3;3	81 3;3	81 3;3
	Ref 4;4	Ref 4;4	Ref 3;3	Ref 2;2	Ref 3;2	Ref 3;3	Ref 3;4	Ref 4;4	Ref 4;3	Ref 3;4	Ref 4;4	Ref 3;3	Ref 2;2	Ref 2;2	Ref 3;2	Ref 2;2	Ref 2;3	Ref 2;3
Pre-dilution	300	300	300	400	400	400	25	25	25	50	50	50	400	400	400	300	300	300
Lab 7 Ref = 1:25000	1 8;8	1 8;8	1 7;7	1 8;8	1 8;8	1 7;8	1 8;8	1 8;7	1 7;7	1 8;8	1 8;8	1 7;7	1 8;8	1 8;8	1 8;8	1 8;8	1 9;9	1 8;8
	3 6;6	3 6;6	3 6;6	3 6;6	3 6;6	3 6;6	3 6;6	3 6;5	3 5;5	3 6;6	3 7;6	3 6;6	3 6;6	3 6;6	3 6;6	3 7;7	3 7;7	3 6;6
	9 4;5	9 5;5	9 4;4	9 4;5	9 5;5	9 4;4	9 5;4	9 4;4	9 3;4	9 5;5	9 5;5	9 4;4	9 5;5	9 4;5	9 4;4	9 5;5	9 5;6	9 5;5
	27 3;3	27 3;3	27 2;3	27 3;3	27 3;3	27 2;3	27 3;3	27 3;2	27 2;2	27 3;3	27 3;3	27 2;2	27 3;3	27 3;3	27 2;3	27 3;3	27 4;4	27 3;3
	81 1;1	81 1;2	81 1;1	81 1;1	81 2;1	81 1;1	81 1;1	81 1;1	81 1;1	81 1;1	81 2;2	81 1;1	81 2;2	81 1;1	81 1;1	81 2;2	81 2;2	81 2;2
	Ref 4;4	Ref 4;5	Ref 5;5	Ref 4;4	Ref 5;5	Ref 5;5	Ref 4;4	Ref 5;5	Ref 4;5	Ref 4;4	Ref 5;5	Ref 5;5	Ref 4;5	Ref 5;5	Ref 5;5	Ref 4;4	Ref 5;5	Ref 5;5

	TxA			TxB			TxC			TxD			TxE			TxF		
	Assay 1	Assay 2	Assay 3	Assay 1	Assay 2	Assay 3	Assay 1	Assay 2	Assay 3	Assay 1	Assay 2	Assay 3	Assay 1	Assay 2	Assay 3	Assay 1	Assay 2	Assay 3
Pre-dilution	250	250	250	250	250	250	15	15	15	15	15	15	500	500	500	500	500	500
Lab 8 Ref = 1:16875	1 8;8	1 9;8	1 7;7	1 8;7	1 7;7	1 7;7	1 8;8	1 7;6	1 7;8	1 8;8	1 8;7	1 9;9	1 8;7	1 8;8	1 8;8	1 8;8	1 8;7	1 9;8
	3 6;6	3 7;7	3 6;6	3 6;5	3 6;6	3 6;5	3 6;6	3 5;5	3 6;6	3 7;7	3 7;6	3 8;8	3 6;5	3 6;6	3 7;7	3 6;6	3 7;6	3 7;7
	9 5;5	9 6;5	9 4;4	9 4;4	9 4;4	9 4;4	9 5;5	9 4;3	9 5;5	9 5;5	9 5;4	9 6;6	9 5;4	9 5;5	9 5;5	9 5;5	9 5;4	9 5;5
	27 3;3	27 4;4	27 4;3	27 3;2	27 2;2	27 2;2	27 3;3	27 2;1	27 3;3	27 3;3	27 3;3	27 5;5	27 3;2	27 3;3	27 3;3	27 3;3	27 3;3	27 4;3
	81 2;2	81 2;2	81 2;2	81 1;1	81 1;1	81 1;1	81 2;2	81 L;L	81 2;2	81 2;2	81 2;1	81 3;4	81 1;1	81 2;1	81 1;2	81 1;1	81 2;1	81 3;2
	Ref 6;6	Ref 6;6	Ref 5;5	Ref 6;5	Ref 6;6	Ref 6;6	Ref 6;6	Ref 6;6	Ref 5;5	Ref 6;5	Ref 6;6	Ref 6;6	Ref 6;5	Ref 6;6	Ref 6;6	Ref 6;6	Ref 6;5	Ref 6;6
Pre-dilution	40	40	40	40	40	40	2	2	2	6	6	6	60	60	60	40	40	40
Lab 9 Ref = 1:2500	1 6;6	1 6;6	1 5;5	1 6;6	1 6;5	1 5;5	1 5;5	1 5;5	1 5;5	1 4;4	1 5;6	1 5;5	1 6;6	1 6;6	1 5;5	1 6;5	1 6;6	1 5;5
	3 5;5	3 5;5	3 4;4	3 5;5	3 5;4	3 4;4	3 4;4	3 4;4	3 4;4	3 3;3	3 4;4	3 4;4	3 5;5	3 5;5	3 4;4	3 4;4	3 5;5	3 4;4
	9 4;4	9 4;4	9 3;3	9 4;4	9 4;3	9 3;3	9 3;3	9 3;3	9 3;3	9 2;2	9 3;3	9 3;3	9 4;3	9 3;4	9 3;3	9 3;3	9 3;3	9 3;3
	27 3;3	27 3;3	27 2;2	27 3;3	27 3;2	27 2;2	27 2;2	27 2;2	27 2;2	27 1;1	27 2;2	27 2;2	27 2;2	27 2;2	27 2;2	27 2;2	27 2;2	27 2;2
	81 2;2	81 2;2	81 1;1	81 2;2	81 2;2	81 1;1	81 1;1	81 1;1	81 1;1	81 L;L	81 1;1	81 1;1	81 1;1	81 1;1	81 1;1	81 1;1	81 1;2	81 1;1
	Ref 4;4	Ref 5;4	Ref 4;4	Ref 4;4	Ref 5;4	Ref 4;4	Ref 4;4	Ref 4;4	Ref 4;4	Ref 4;4	Ref 4;5	Ref 4;4	Ref 4;4	Ref 4;4	Ref 4;4	Ref 4;4	Ref 4;4	Ref 4;4
Pre-dilution	500	500	500	500	500	500	25	25	25	50	50	50	500	500	500	500	500	500
Lab 10 Ref = 1:30000	1 8;8	1 8;8	1 8;8	1 9;9	1 8;8	1 7;7	1 8;8	1 8;8	1 8;7	1 8;8	1 8;8	1 8;8	1 9;8	1 8;8	1 8;8	1 8;8	1 8;8	1 8;8
	3 6;7	3 6;6	3 7;6	3 7;7	3 6;6	3 6;6	3 7;7	3 6;6	3 6;5	3 7;7	3 7;7	3 6;6	3 7;7	3 7;7	3 7;6	3 6;6	3 6;6	3 6;6
	9 4;5	9 5;5	9 5;5	9 5;5	9 5;5	9 4;4	9 5;5	9 5;4	9 3;4	9 5;5	9 5;5	9 5;5	9 5;5	9 5;5	9 5;5	9 5;4	9 4;4	9 4;4
	27 3;4	27 3;3	27 3;2	27 4;4	27 3;3	27 3;3	27 4;4	27 3;3	27 2;2	27 4;4	27 3;3	27 3;3	27 4;4	27 3;3	27 3;3	27 3;3	27 3;3	27 3;2
	81 2;2	81 2;2	81 L;1	81 2;2	81 1;1	81 1;1	81 2;2	81 1;1	81 1;1	81 2;2	81 2;2	81 2;1	81 2;2	81 1;2	81 1;2	81 1;1	81 1;1	81 1;1
	Ref 6;6	Ref 6;6	Ref 6;5	Ref 6;6	Ref 5;5	Ref 5;6	Ref 6;6	Ref 5;5	Ref 5;5	Ref 6;6	Ref 5;5	Ref 5;5	Ref 6;6	Ref 5;5	Ref 6;5	Ref 6;7	Ref 5;5	Ref 5;5

	TxA						TxB						TxC						TxD						TxE						TxF					
	Assay 1		Assay 2		Assay 3		Assay 1		Assay 2		Assay 3		Assay 1		Assay 2		Assay 3		Assay 1		Assay 2		Assay 3		Assay 1		Assay 2		Assay 3		Assay 1		Assay 2		Assay 3	
Pre-dilution	20		20		20		10		10		10		4		4		4		4		4		4		20		20		20		20		20		20	
Lab 11 Ref = 1:4000	1	D;D	1	D;D	1	9;D	1	D;D	1	D;D	1	D;D	1	9;9	1	6;7	1	8;7	1	D;9	1	9;D	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D
	3	9;8	3	8;7	3	7;8	3	9;9	3	8;8	3	8;8	3	6;5	3	4;4	3	5;5	3	7;7	3	7;8	3	8;8	3	D;D	3	7;7	3	9;8	3	9;9	3	8;9	3	9;9
	9	6;6	9	6;5	9	5;5	9	7;6	9	5;6	9	6;6	9	3;3	9	3;2	9	3;3	9	5;5	9	5;6	9	5;5	9	7;8	9	5;4	9	7;6	9	6;6	9	5;5	9	7;5
	27	4;3	27	4;3	27	3;3	27	5;4	27	3;3	27	4;3	27	2;1	27	L;L	27	1;1	27	3;3	27	4;4	27	3;3	27	4;4	27	2;2	27	5;4	27	4;3	27	3;3	27	4;3
	81	3;2	81	3;2	81	2;2	81	3;3	81	2;2	81	3;3	81	1;L	81	L;L	81	L;L	81	2;2	81	2;3	81	2;2	81	3;3	81	1;1	81	3;2	81	3;3	81	1;2	81	3;2
	Ref	8;7	Ref	6;6	Ref	5;5	Ref	7;7	Ref	5;5	Ref	6;6	Ref	7;7	Ref	5;5	Ref	6;5	Ref	5;5	Ref	5;5	Ref	5;5	Ref	8;7	Ref	5;5	Ref	5;5	Ref	7;7	Ref	6;5	Ref	5;5

Table C – TCP in vivo full testing. Summary overview of endpoints

	TdG			TdH			TdJ			TdK			TdL			TdM		
	Assay 1	Assay 2	Assay 3	Assay 1	Assay 2	Assay 3	Assay 1	Assay 2	Assay 3	Assay 1	Assay 2	Assay 3	Assay 1	Assay 2	Assay 3	Assay 1	Assay 2	Assay 3
TCP	75	< 60	> 130	30	10	65	10	10	10	70	> 115	> 215	< 25	> 75	155	< 25	> 75	> 215
Lab 1 L^+ = 170	70 D;D	60 L;L	50 D;D	15 D;D	5 D;D	5 D;D	5 D;D	5 D;D	5 D;D	65 D;D	35 D;D	135 D;D	25 L;L	35 D;D	115 D;D	25 L;L	35 D;D	135 D;D
	80 L;L	70 L;L	70 D;D	25 D;D	15 L;L	25 D;D	15 L;L	15 L;L	25 L;L	75 L;L	55 D;D	155 D;D	35 L;L	45 D;D	135 D;L	35 L;L	45 D;D	155 D;D
	90 L;L	80 L;L	90 D;D	35 L;L	25 L;L	45 D;D	25 L;L	25 L;L	45 L;L	85 L;L	75 D;D	175 D;D	45 L;L	55 D;D	155 D;L	45 L;L	55 D;D	175 D;D
	100 L;L	90 L;L	110 D;D	45 L;L	35 L;L	65 D;L	35 L;L	35 L;L	65 L;L	95 L;L	95 D;D	195 D;D	55 L;L	65 D;D	175 D;L	55 L;L	65 D;D	195 D;D
	110 L;L	100 L;L	130 D;D	55 L;L	45 L;L	85 L;L	45 L;L	45 L;L	85 L;L	105 L;L	115 D;D	215 D;D	65 L;L	75 D;D	195 L;L	65 L;L	75 D;D	215 D;D
TCP	110	90	70	50	30	30	20	20	< 10	140	120	90	60	20	10	40	20	20
Lab 2 L^+ = 170	40 D;D	40 D;D	40 D;D	20 D;D	20 D;D	20 D;D	10 D;D	10 D;D	10 L;L	80 D;D	80 D;D	80 D;D	10 D;D	10 D;D	10 D;L	10 D;D	10 D;D	10 D;D
	60 D;D	60 D;D	60 D;D	40 D;D	40 L;L	40 L;L	30 L;L	30 L;L	30 L;L	100 D;D	100 D;D	100 L;L	30 D;D	30 L;L	30 L;L	30 D;D	30 L;L	30 L;L
	80 D;D	80 D;D	80 L;L	60 L;L	60 L;L	60 L;L	50 L;L	50 L;L	50 L;L	120 D;D	120 D;L	120 L;L	50 D;D	50 L;L	50 L;L	50 L;L	50 L;L	50 L;L
	100 D;D	100 L;L	100 L;L	80 L;L	80 L;L	80 L;L	70 L;L	70 L;L	70 L;L	140 D;L	140 L;L	140 L;L	70 L;L	70 L;L	70 L;L	70 L;L	70 L;L	70 L;L
	120 L;L	120 L;L	120 L;L	100 L;L	100 L;L	100 L;L	90 L;L	90 L;L	90 L;L	160 L;L	160 L;L	160 L;L	90 L;L	90 L;L	90 L;L	90 L;L	90 L;L	90 L;L
TCP	150	180	200	50	60	50	30	50	50	> 140	> 200	> 180	> 80	90	130	INV	INV	INV
Lab 3 L^+ = 170	100 D;D	100 D;D	120 D;D	2 D;D	2 D;D	20 D;D	2 D;D	2 D;D	2 D;D	60 D;D	120 D;D	100 D;D	2 D;D	20 D;D	60 D;D	2 D;D	20 D;D	20 D;D
	120 D;D	120 D;D	140 D;D	20 D;D	20 D;D	40 D;D	20 D;D	20 D;D	20 D;D	80 D;D	140 D;D	120 D;D	20 D;D	40 D;D	80 D;D	20 D;D	40 D;D	40 D;D
	140 D;D	140 D;D	160 D;D	40 D;D	40 D;D	60 L;L	40 L;L	40 D;D	40 D;D	100 D;D	160 D;D	140 D;D	40 D;D	60 D;D	100 D;D	40 D;D	60 L;L	60 L;L
	160 L;L	160 D;D	180 D;D	60 L;L	60 L;D	80 L;L	60 L;L	60 L;L	60 L;L	120 D;D	180 D;D	160 D;D	60 D;D	80 D;D	120 L;D	60 L;D	80 L;D	80 L;D
	180 L;L	180 L;D	200 L;D	80 L;L	80 L;L	100 L;L	80 L;L	80 L;L	80 L;L	140 D;D	200 D;D	180 D;D	80 D;D	100 L;L	140 L;D	80 D;D	100 L;L	100 L;L
TCP	80	130	150	40	40	60	20	20	20	200	190	170	70	80	60	70	40	90
Lab 4 L^+ = 170	80 D;L	80 D;D	80 D;D	10 D;D	10 D;D	10 D;D	10 D;D	10 D;D	10 D;D	130 D;D	130 D;D	130 D;D	40 D;D	40 D;D	40 D;D	20 D;D	20 D;D	20 D;D
	100 L;L	100 D;D	100 D;D	30 D;D	30 D;D	30 D;D	30 L;L	30 L;L	30 L;L	150 D;D	150 D;D	150 D;D	60 D;D	60 D;D	60 D;L	40 D;D	40 D;L	40 D;D
	120 L;L	120 D;D	120 D;D	50 L;L	50 L;L	50 D;D	50 L;L	50 L;L	50 L;L	170 D;D	170 D;D	170 D;L	80 L;L	80 D;L	80 L;L	60 D;L	60 L;L	60 D;D
	140 L;L	140 L;L	140 D;D	70 L;L	70 L;L	70 L;L	70 L;L	70 L;L	70 L;L	190 D;D	190 D;L	190 L;L	100 L;L	100 L;L	100 X;X	80 D;L	80 L;L	80 D;L
	160 L;L	160 L;L	160 L;L	90 L;L	90 L;L	90 L;L	90 L;L	90 L;L	90 L;L	210 L;L	210 L;L	210 L;L	120 L;L	120 L;L	120 L;L	100 L;L	100 L;L	100 D;L

	TdG			TdH			TdJ			TdK			TdL			TdM		
	Assay 1	Assay 2	Assay 3	Assay 1	Assay 2	Assay 3	Assay 1	Assay 2	Assay 3	Assay 1	Assay 2	Assay 3	Assay 1	Assay 2	Assay 3	Assay 1	Assay 2	Assay 3
TCP	170	180	200	80	80	60	30	40	20	250	210	200	100	70	70	90	60	70
Lab 5 L^+ = 133.3	140 D;D	140 D;D	140 D;D	50 D;D	50 D;D	50 D;D	10 D;D	10 D;D	10 D;D	190 D;D	190 D;D	190 D;D	60 D;D	60 D;D	60 D;D	50 D;D	50 D;D	50 D;D
	160 D;D	160 D;L	160 D;D	70 D;D	70 D;D	70 L;L	30 D;L	30 D;D	30 L;L	210 D;D	210 D;L	210 L;L	80 D;D	80 L;L	80 L;L	70 D;L	70 L;L	70 D;L
	180 L;L	180 D;L	180 D;D	90 L;L	90 L;L	90 L;L	50 L;L	50 L;L	50 L;L	230 D;D	230 L;L	230 L;L	100 D;L	100 L;L	100 L;L	90 D;L	90 L;L	90 L;L
	200 L;L	200 D;L	200 D;L	110 L;L	110 L;L	110 L;L	70 L;L	70 L;L	70 L;L	250 D;L	250 L;L	250 L;L	120 L;L	120 L;L	120 L;L	110 D;L	110 L;L	110 L;L
	220 L;L	220 L;L	220 L;L	130 L;L	130 L;L	130 L;L	90 L;L	90 L;L	90 L;L	270 L;L	270 L;L	270 L;L	140 L;L	140 L;L	140 L;L	130 L;L	130 L;L	130 L;L
TCP	170	180	170	80	80	70	20	20	20	210	210	220	120	120	110	220	220	220
Lab 6 L^+ = 143	100 D;D	100 D;D	100 D;D	10 D;D	10 D;D	10 D;D	– –	– –	– –	150 D;D	150 D;D	150 D;D	60 D;D	60 D;D	60 D;D	140 D;D	140 D;D	140 D;D
	120 D;D	120 D;D	120 D;D	30 D;D	30 D;D	30 D;D	– –	– –	– –	170 D;D	170 D;D	170 D;D	80 D;D	80 D;D	80 D;D	160 D;D	160 D;D	160 D;D
	140 D;D	140 D;D	140 D;D	50 D;D	50 D;D	50 D;D	10 D;D	10 D;D	10 D;D	190 D;D	190 D;D	190 D;D	100 D;D	100 D;D	100 D;D	180 D;D	180 D;D	180 D;D
	160 D;D	160 D;D	160 D;D	70 D;D	70 D;D	70 D;L	30 L;L	30 L;L	30 L;L	210 D;L	210 D;L	210 D;D	120 D;L	120 D;L	120 L;L	200 D;D	200 D;D	200 D;D
	180 L;L	180 D;L	180 L;L	90 L;L	90 L;L	90 L;L	50 L;L	50 L;L	50 L;L	230 L;L	230 L;L	230 L;L	140 L;L	140 L;L	140 L;L	220 D;L	220 D;L	220 D;L

Table D – TCP in vitro full testing. Summary overview of endpoints

	TdG						TdH						TdJ						TdK						TdL						TdM						Antitoxin/toxin Control					
	Assay 1		Assay 2		Assay 3		Assay 1		Assay 2		Assay 3		Assay 1		Assay 2		Assay 3		Assay 1		Assay 2		Assay 3		Assay 1		Assay 2		Assay 3		Assay 1		Assay 2		Assay 3		Assay 1		Assay 2		Assay 3	
Pre-dilution	1		1		1		1		1		1		1		1		1		1		1		1		1		1		1		1		1		1		1		1		1	
Lab 2 L^+ = 170	40	D;D	40	D;D	40	D;D	20	D;D	20	D;D	20	9;9	10	D;D	10	D;D	10	8;8	80	D;D	80	D;D	80	9;9	10	D;D	10	D;D	10	D;D	10	D;D	10	9;9	1.50	8;8	1.50	7;7	1.50	7;7
	60	D;D	60	D;D	60	9;9	40	D;D	40	D;D	40	9;9	30	8;8	30	8;8	30	7;7	100	D;D	100	D;D	100	9;9	30	D;D	30	D;D	30	9;9	30	D;D	30	D;D	30	8;8	1.25	9;9	1.25	8;8	1.25	8;8
	80	D;D	80	D;D	80	9;9	60	9;9	60	9;9	60	8;8	50	7;7	50	7;7	50	6;6	120	D;D	120	D;D	120	9;9	50	D;D	50	D;D	50	9;9	50	D;D	50	9;9	50	8;7	1.00	D;D	1.00	9;9	1.00	8;8
	100	D;D	100	D;D	100	8;8	80	8;8	80	8;8	80	8;8	70	6;6	70	6;6	70	6;5	140	D;D	140	9;9	140	8;8	70	D;D	70	9;9	70	8;8	70	D;9	70	8;8	70	7;7	0.75	D;D	0.75	9;9	0.75	9;9
	120	D;D	120	9;9	120	8;8	100	7;7	100	8;7	100	7;7	90	6;6	90	6;5	90	5;5	160	9;9	160	9;8	160	8;8	90	9;D	90	9;8	90	8;8	90	9;8	90	8;8	90	7;6	0.50	D;D	0.50	D;D	0.50	9;9
Pre-dilution	4		4		4		4		4		4		4		4		4		4		4		4		4		4		4		4		4		4		4		4	
Lab 3a L^+ = 170	100	9;X	100	9;9	100	9;9	2	9;9	2	9;D	20	9;9	2	9;D	2	D;D	2	9;9	60	9;9	120	9;9	100	9;D	2	9;9	20	8;8	60	8;8	2	9;D	20	8;8	20	9;9	1.50	6;6	1.50	8;7	1.50	6;6
	120	8;X	120	8;9	120	8;8	20	D;D	20	D;D	40	8;9	20	8;D	20	D;D	20	8;8	80	9;8	140	9;9	120	9;D	20	9;9	40	8;8	80	8;8	20	9;9	40	7;8	40	8;9	1.25	7;7	1.25	8;8	1.25	7;7
	140	8;X	140	8;9	140	8;8	40	8;8	40	8;9	60	8;8	40	7;.	40	8;8	40	7;7	100	8;8	160	8;8	140	9;9	40	9;9	60	8;8	100	7;7	40	7;8	60	7;7	60	8;8	1.00	7;8	1.00	9;9	1.00	6;8
	160	8;X	160	8;9	160	8;8	60	6;6	60	7;8	80	7;7	60	6;6	60	7;7	60	6;6	120	8;8	180	8;8	160	9;9	60	8;8	80	7;7	120	7;6	60	8;8	80	7;7	80	8;8	0.75	D;9	0.75	9;9	0.75	6;8
	180	7;X	180	8;8	180	8;8	80	6;6	80	7;7	100	7;7	80	6;5	80	7;7	80	6;6	140	8;8	200	8;8	180	9;8	80	7;8	100	6;7	140	7;7	80	8;8	100	7;7	100	8;8	0.50	D;D	0.50	D;D	0.50	7;9
Pre-dilution	4		4		4		4		4		4		4		4		4		4		4		4		4		4		4		4		4		4		4		4	
Lab 3b L^+ = 170	100	6;7	100	8;9	100	9;9	2	7;7	2	9;9	20	9;9	2	9;D	2	D;D	2	9;D	60	8;9	120	9;9	100	7;7	2	9;9	20	8;8	60	8;8	2	9;9	20	8;8	20	X;7	1.50	–	1.50	7;7	1.50	5;4
	120	6;7	120	8;9	120	8;8	20	7;7	20	9;9	40	8;8	20	8;D	20	9;9	20	8;8	80	9;8	140	9;9	120	7;6	20	9;9	40	8;8	80	6;6	20	8;9	40	7;8	40	6;6	1.25	–	1.25	7;7	1.25	5;5
	140	6;6	140	8;8	140	8;8	40	7;5	40	8;9	60	7;8	40	7;X	40	8;8	40	7;7	100	8;8	160	8;8	140	6;6	40	9;9	60	7;7	100	5;5	40	7;8	60	7;7	60	5;5	1.00	–	1.00	8;8	1.00	5;5
	160	5;6	160	8;X	160	8;8	60	4;4	60	7;8	80	7;7	60	6;6	60	7;X	60	6;5	120	8;8	180	8;X	160	6;6	60	8;8	80	7;7	120	5;5	60	7;8	80	7;7	80	5;5	0.75	–	0.75	9;9	0.75	6;6
	180	5;5	180	6;X	180	7;8	80	4;4	80	7;7	100	6;6	80	5;5	80	7;7	80	6;4	140	8;8	200	8;8	180	6;6	80	7;8	100	6;7	140	5;5	80	7;7	100	7;7	100	5;5	0.50	–	0.50	9;9	0.50	7;7

	TdG			TdH			TdJ			TdK			TdL			TdM			Antitoxin/toxin Control		
	Assay 1	Assay 2	Assay 3	Assay 1	Assay 2	Assay 3	Assay 1	Assay 2	Assay 3	Assay 1	Assay 2	Assay 3	Assay 1	Assay 2	Assay 3	Assay 1	Assay 2	Assay 3	Assay 1	Assay 2	Assay 3
Pre-dilution	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16
Lab 4 L^+ = 170	80 5;5	80 5;5	80 6;6	10 5;5	10 6;6	10 5;5	10 5;5	10 6;5	10 5;6	130 5;5	130 5;5	130 5;5	40 5;5	40 5;5	40 5;5	20 4;4	20 4;4	20 5;5	1.50 3;3	1.50 2;3	1.50 2;2
	100 5;5	100 5;5	100 6;6	30 5;5	30 5;6	30 5;5	30 4;4	30 4;4	30 4;4	150 4;4	150 5;4	150 5;5	60 4;4	60 5;5	60 4;5	40 4;3	40 4;4	40 5;4	1.25 3;4	1.25 3;3	1.25 4;4
	120 4;5	120 5;5	120 5;5	50 4;4	50 5;5	50 4;4	50 2;2	50 3;3	50 3;3	170 4;4	170 4;4	170 4;5	80 4;4	80 4;4	80 4;4	60 3;3	60 4;4	60 4;4	1.00 4;4	1.00 4;4	1.00 4;4
	140 4;4	140 4;4	140 5;5	70 3;3	70 4;4	70 3;3	70 2;1	70 2;2	70 2;2	190 4;4	190 4;4	190 4;4	100 3;4	100 4;4	100 4;4	80 3;3	80 3;3	80 4;4	0.75 5;5	0.75 5;5	0.75 5;5
	160 4;4	160 4;4	160 5;5	90 2;2	90 4;4	90 3;3	90 L;L	90 2;2	90 2;2	210 3;4	210 4;4	210 4;4	120 3;3	120 4;4	120 3;3	100 3;X	100 3;3	100 4;4	0.50 5;5	0.50 5;5	0.50 6;6
Pre-dilution	64	64	64	64	64	64	64	64	64	64	64	64	64	64	64	64	64	64	64	64	64
Lab 5 L^+ = 133.3	10 5;5	10 5;5	10 5;5	10 5;5	10 5;5	10 5;5	10 4;4	10 5;5	10 4;4	60 5;5	60 5;5	60 5;5	10 5;5	10 5;5	10 5;5	30 5;5	30 5;5	30 5;5	1.50 4;4	1.50 3;3	1.50 3;4
	30 5;5	30 5;5	30 5;5	30 4;4	30 4;4	30 4;3	30 3;3	30 3;4	30 3;3	80 5;5	80 5;5	80 5;5	30 5;5	30 5;5	30 5;5	50 4;4	50 4;5	50 5;5	1.25 4;4	1.25 4;4	1.25 4;4
	50 5;5	50 4;4	50 5;5	50 4;4	50 4;4	50 3;3	50 3;3	50 3;3	50 2;2	100 4;4	100 5;5	100 4;4	50 4;4	50 5;5	50 4;4	70 4;4	70 4;4	70 4;4	1.00 5;4	1.00 4;5	1.00 4;4
	70 4;5	70 4;4	70 5;5	70 3;4	70 3;3	70 3;3	70 3;3	70 3;3	70 2;2	120 4;4	120 5;4	120 4;4	70 4;4	70 4;5	70 4;4	90 4;4	90 4;4	90 4;4	0.75 5;5	0.75 5;5	0.75 5;5
	90 4;4	90 4;4	90 4;4	90 3;4	90 3;3	90 3;3	90 3;3	90 3;3	90 2;2	140 4;4	140 4;4	140 4;4	90 4;4	90 4;4	90 4;4	110 4;4	110 4;4	110 4;4	0.50 5;5	0.50 5;5	0.50 5;5
Pre-dilution	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16
Lab 6 L^+ = 143	100 8;8	100 6;6	100 6;6	10 8;8	10 7;7	10 6;7	10 7;6	10 7;7	10 7;7	150 6;6	150 5;5	150 7;6	60 6;6	60 6;6	60 7;7	140 5;5	140 4;4	140 6;6	1.50 6;6	1.50 3;3	1.50 4;4
	120 8;8	120 6;6	120 6;6	30 8;8	30 6;6	30 6;6	30 5;5	30 6;6	30 6;6	170 6;6	170 5;5	170 6;6	80 5;6	80 6;5	80 6;7	160 4;4	160 4;4	160 6;6	1.25 6;6	1.25 4;4	1.25 5;5
	140 7;7	140 6;6	140 6;6	50 7;7	50 6;6	50 5;5	50 5;4	50 5;6	50 5;6	190 6;5	190 5;5	190 6;6	100 5;5	100 5;5	100 6;7	180 4;4	180 4;4	180 6;6	1.00 7;7	1.00 5;5	1.00 5;5
	160 7;7	160 6;5	160 5;5	70 7;7	70 5;5	70 5;5	999 3;2	999 3;3	999 4;4	210 5;5	210 5;5	210 6;6	120 5;5	120 4;4	120 6;7	200 4;5	200 4;4	200 5;5	0.75 7;7	0.75 6;6	0.75 6;6
	180 7;7	180 6;5	180 5;5	90 6;6	90 5;5	90 4;4	– –	– –	– –	230 5;5	230 5;5	230 6;6	140 5;5	140 4;4	140 7;7	220 4;5	220 4;4	220 5;6	0.50 8;8	0.50 6;6	0.50 6;6
	999 5;5	999 1;2	999 3;2	999 5;5	999 2;2	999 2;2	– –	– –	– –	999 2;2	999 L;L	999 4;4	999 2;2	999 L;L	999 4;4	999 2;2	999 1;1	999 4;4	– –	– –	– –
Pre-dilution	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
Lab 7 L^+ = 170	60 7;7	60 7;7	60 7;7	10 7;7	10 7;7	10 7;7	– –	– –	– –	110 6;6	110 6;7	110 6;7	20 6;6	20 6;6	20 6;7	20 6;6	20 6;6	20 7;6	1.50 4;4	1.50 3;3	1.50 3;3
	80 6;7	80 7;7	80 6;7	30 6;6	30 6;7	30 6;6	– –	– –	– –	130 6;6	130 6;6	130 6;6	40 6;6	40 6;6	40 6;6	40 6;6	40 6;6	40 6;6	1.25 4;4	1.25 4;4	1.25 4;4
	100 6;6	100 6;6	100 6;6	50 5;5	50 6;5	50 5;5	10 7;7	10 7;7	10 7;7	150 6;6	150 6;6	150 6;6	60 6;6	60 6;6	60 6;6	60 5;5	60 5;5	60 5;5	1.00 5;5	1.00 5;5	1.00 5;5
	120 6;6	120 6;6	120 6;6	70 5;5	70 5;5	70 5;5	30 5;5	30 5;5	30 5;5	170 5;6	170 6;6	170 6;5	80 5;6	80 6;6	80 6;5	80 5;5	80 5;5	80 5;5	0.75 6;6	0.75 6;6	0.75 6;6
	140 6;6	140 6;6	140 6;6	90 4;4	90 4;4	90 4;4	50 4;4	50 4;4	50 4;4	190 5;5	190 5;5	190 5;5	100 5;5	100 5;5	100 5;5	100 5;5	100 5;5	100 5;5	0.50 7;7	0.50 7;7	0.50 7;6

	TdG			TdH			TdJ			TdK			TdL			TdM			Antitoxin/toxin Control		
	Assay 1	Assay 2	Assay 3	Assay 1	Assay 2	Assay 3	Assay 1	Assay 2	Assay 3	Assay 1	Assay 2	Assay 3	Assay 1	Assay 2	Assay 3	Assay 1	Assay 2	Assay 3	Assay 1	Assay 2	Assay 3
Pre-dilution	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	1	1	1
Lab 8 L^+ = 170	40 6;7	40 6;7	40 6;6	20 7;7	20 7;6	20 6;6	20 6;6	20 5;5	20 5;5	120 6;6	120 5;5	120 5;6	20 6;6	20 5;5	20 6;5	100 5;5	100 5;5	100 5;5	1.50 8;9	1.50 7;7	1.50 8;8
	60 6;7	60 6;7	60 6;7	40 6;7	40 6;6	40 6;6	40 5;5	40 5;5	40 4;4	140 6;6	140 5;5	140 5;6	40 6;6	40 5;5	40 6;5	120 5;5	120 5;5	120 4;4	1.25 9;9	1.25 8;8	1.25 9;9
	80 6;7	80 6;7	80 7;7	60 6;6	60 5;6	60 6;6	60 5;5	60 5;4	60 4;4	160 6;6	160 5;5	160 5;6	60 6;6	60 5;5	60 5;5	140 5;5	140 5;5	140 4;4	1.00 9;D	1.00 8;8	1.00 9;9
	100 6;7	100 6;7	100 7;7	80 6;6	80 5;5	80 6;6	80 5;5	80 5;4	80 4;4	180 6;6	180 5;5	180 5;5	80 6;6	80 5;5	80 5;5	160 5;5	160 5;4	160 4;4	0.75 D;D	0.75 9;9	0.75 D;D
	120 6;6	120 6;6	120 6;7	100 5;5	100 5;5	100 6;5	100 4;5	100 4;4	100 3;3	200 6;6	200 5;5	200 5;5	100 6;6	100 5;5	100 5;5	180 5;5	180 5;4	180 4;4	0.50 D;D	0.50 9;9	0.50 D;D
Pre-dilution	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Lab 9 L^+ = 170	60 D;D	60 D;D	60 9;9	10 D;D	10 D;D	10 D;D	2 D;D	2 D;D	2 D;D	110 9;9	110 9;9	110 9;9	20 9;9	20 9;9	20 9;9	2 9;9	2 D;D	2 D;D	1.50 6;6	1.50 6;6	1.50 5;5
	80 9;D	80 9;9	80 9;9	30 9;9	30 9;D	30 9;9	4 D;D	4 D;D	4 D;D	130 9;8	130 8;8	130 8;8	40 9;9	40 8;8	40 9;9	4 9;9	4 9;9	4 9;D	1.25 7;7	1.25 7;7	1.25 6;6
	100 9;9	100 9;9	100 8;8	50 8;9	50 8;D	50 8;7	10 D;D	10 D;D	10 9;9	150 9;8	150 8;8	150 8;8	60 9;9	60 8;8	60 8;8	20 7;7	20 8;8	20 8;9	1.00 8;8	1.00 8;8	1.00 7;7
	120 8;8	120 8;8	120 8;8	70 8;8	70 7;D	70 7;7	30 8;8	30 8;X	30 7;7	170 9;8	170 8;8	170 8;8	80 8;8	80 7;7	80 7;7	40 L;L	40 7;8	40 8;8	0.75 9;8	0.75 8;8	0.75 8;8
	140 8;8	140 8;8	140 7;7	90 7;7	90 6;7	90 6;6	50 7;7	50 7;7	50 6;6	190 8;8	190 L;L	190 7;7	100 7;7	100 7;7	100 7;7	60 L;L	60 7;8	60 7;7	0.50 9;9	0.50 9;9	0.50 9;9
Pre-dilution	16	8	8	16	8	8	16	8	8	16	8	8	16	8	8	16	8	8	16	8	8
Lab 10 L^+ = 170	10 6;6	120 6;6	160 6;6	10 6;6	40 6;6	40 7;6	10 5;5	10 7;7	10 6;7	10 6;6	80 7;7	160 6;6	10 6;6	40 6;6	80 6;6	10 6;6	40 6;6	40 6;5	1.50 3;3	1.50 4;4	1.50 4;3
	30 6;6	140 6;6	180 6;6	30 6;6	60 6;5	60 5;6	30 5;4	30 5;5	30 6;5	30 6;6	100 6;6	180 6;6	30 6;6	60 6;6	100 6;6	30 5;5	60 6;6	60 5;5	1.25 4;4	1.25 5;5	1.25 5;5
	50 6;6	160 6;6	200 6;6	50 6;5	80 5;5	80 4;5	50 4;4	50 4;4	50 5;4	50 6;6	120 6;6	200 6;6	50 6;6	80 6;6	120 5;6	50 5;5	80 6;5	80 5;5	1.00 5;5	1.00 5;5	1.00 5;5
	70 6;6	180 6;6	220 6;5	70 5;5	100 5;5	100 4;5	70 4;3	70 4;4	70 5;4	70 6;6	140 6;6	220 6;6	70 6;6	100 6;6	140 5;5	70 5;5	100 5;5	100 5;5	0.75 5;5	0.75 6;6	0.75 6;6
	90 6;6	200 6;5	240 6;6	90 5;5	120 5;4	120 3;5	90 3;3	90 3;4	90 5;3	90 5;6	160 6;6	240 5;5	90 5;5	120 6;5	160 4;4	90 5;5	120 5;5	120 5;5	0.50 5;6	0.50 6;6	0.50 6;6
	999 1;1	999 L;L	999 L;L	999 2;1	999 L;L	999 L;L	999 L;1	999 L;L	999 L;L	999 2;1	999 L;L	999 L;L	999 1;1	999 L;L	999 L;L	999 L;1	999 L;L	999 L;L	999 1;1	999 L;L	999 L;L
Pre-dilution	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
Lab 11 L^+ = 170	120 4;4	120 5;6	120 5;6	10 5;5	10 5;5	10 6;6	10 5;5	10 5;5	10 6;7	100 4;4	100 5;4	100 6;6	20 6;5	20 6;5	20 6;5	20 4;4	20 3;4	20 5;7	1.50 L;L	1.50 L;L	1.50 L;L
	140 3;3	140 4;5	140 5;6	30 5;5	30 5;5	30 5;5	30 3;4	30 3;4	30 5;4	120 4;4	120 5;4	120 5;5	40 5;5	40 5;4	40 5;4	40 4;4	40 L;2	40 6;6	1.25 3;3	1.25 3;3	1.25 2;2
	160 2;1	160 3;3	160 5;5	50 3;3	50 L;L	50 L;L	50 L;L	50 L;L	50 L;L	140 3;3	140 4;3	140 6;4	60 4;4	60 3;3	60 4;3	60 2;2	60 L;L	60 6;4	1.00 3;3	1.00 3;4	1.00 3;3
	180 L;L	180 L;L	180 4;5	70 L;L	70 L;L	70 L;L	70 L;L	70 L;L	70 L;L	160 1;2	160 3;3	160 4;3	80 3;3	80 L;L	80 2;2	80 L;L	80 L;L	80 6;5	0.75 4;3	0.75 4;4	0.75 4;4
	200 L;L	200 L;L	200 4;5	90 L;L	90 L;L	90 L;L	90 L;L	90 L;L	90 L;L	180 L;L	180 2;2	180 3;4	100 3;1	100 L;L	100 L;L	100 L;L	100 L;L	100 L;L	0.50 5;4	0.50 4;5	0.50 4;5

Table E – VI test – summary overview of endpoints

	Antitoxin/Toxin Control											
	Assay 1		Assay 2		Assay 3		Assay 4		Assay 5		Assay 6	
Pre-dilution	1		1		1							
Lab 2 L^+ = 170	1.50	8;8	1.50	7;7	1.50	7;7						
	1.25	9;9	1.25	8;8	1.25	8;8						
	1.00	D;D	1.00	9;9	1.00	8;8						
	0.75	D;D	0.75	9;9	0.75	9;9						
	0.50	D;D	0.50	D;D	0.50	9;9						
Pre-dilution	4		4		4							
Lab 3a L^+ = 170	1.50	6;6	1.50	8;7	1.50	6;6						
	1.25	7;7	1.25	8;8	1.25	7;7						
	1.00	7;8	1.00	9;9	1.00	6;8						
	0.75	D;9	0.75	9;9	0.75	6;8						
	0.50	D;D	0.50	D;D	0.50	7;9						
Pre-dilution	4		4		4		4		4		4	
Lab 3b L^+ = 170	1.50	–	1.50	7;7	1.50	5;4	1.50	7;7	1.50	6;6	1.50	4;X
	1.25	–	1.25	7;7	1.25	5;5	1.25	7;7	1.25	8;7	1.25	5;5
	1.00	–	1.00	8;8	1.00	5;5	1.00	9;8	1.00	8;9	1.00	8;7
	0.75	–	0.75	9;9	0.75	6;6	0.75	D;9	0.75	9;9	0.75	9;9
	0.50	–	0.50	9;9	0.50	7;7	0.50	D;D	0.50	D;D	0.50	D;D
Pre-dilution	16		16		16		16		16		16	
Lab 4 L^+ = 170	1.50	3;3	1.50	2;3	1.50	2;2	1.50	2;2	1.50	3;2	1.50	2;2
	1.25	3;4	1.25	3;3	1.25	4;4	1.25	3;3	1.25	4;4	1.25	3;3
	1.00	4;4	1.00	4;4	1.00	4;4	1.00	4;4	1.00	5;4	1.00	4;4
	0.75	5;5	0.75	5;5	0.75	5;5	0.75	4;5	0.75	5;5	0.75	5;5
	0.50	5;5	0.50	5;5	0.50	6;6	0.50	5;5	0.50	6;6	0.50	5;5

	Antitoxin/Toxin Control															
	Assay 1		Assay 2		Assay 3		Assay 4		Assay 5		Assay 6		Assay 7		Assay 8	
Pre-dilution	64		64		64		64		64		64		64			
Lab 5 L^+ = 133.3	1.50	4;4	1.50	3;3	1.50	3;4	1.50	4;4	1.50	4;4	1.50	4;4	1.50	4;4		
	1.25	4;4	1.25	4;4	1.25	4;4	1.25	5;4	1.25	4;4	1.25	5;4	1.25	4;4		
	1.00	5;4	1.00	4;5	1.00	4;4	1.00	5;5	1.00	4;4	1.00	5;5	1.00	4;4		
	0.75	5;5	0.75	5;5	0.75	5;5	0.75	5;5	0.75	5;5	0.75	5;5	0.75	5;5		
	0.50	5;5	0.50	5;5	0.50	5;5	0.50	6;6	0.50	5;5	0.50	6;6	0.50	5;5		
Pre-dilution	64		64		64		64		64		64		64		64	
Lab 6 L^+ = 143	1.50	6;6	1.50	3;3	1.50	4;4	1.50	4;4	1.50	3;4	1.50	5;4	1.50	5;5	1.50	5;5
	1.25	6;6	1.25	4;4	1.25	5;5	1.25	5;5	1.25	4;4	1.25	5;5	1.25	6;6	1.25	6;6
	1.00	7;7	1.00	5;5	1.00	5;5	1.00	5;5	1.00	4;5	1.00	6;6	1.00	6;6	1.00	6;6
	0.75	7;7	0.75	6;6	0.75	6;6	0.75	6;6	0.75	5;5	0.75	6;6	0.75	7;7	0.75	7;7
	0.50	8;8	0.50	6;6	0.50	6;6	0.50	6;6	0.50	5;5	0.50	7;7	0.50	7;7	0.50	7;7
Pre-dilution	8		8		8											
Lab 7 L^+ = 170	1.50	4;4	1.50	3;3	1.50	3;3										
	1.25	4;4	1.25	4;4	1.25	4;4										
	1.00	5;5	1.00	5;5	1.00	5;5										
	0.75	6;6	0.75	6;6	0.75	6;6										
	0.50	7;7	0.50	7;7	0.50	7;6										
Pre-dilution	1		1		1											
Lab 8 L^+ = 170	1.50	8;9	1.50	7;7	1.50	8;8										
	1.25	9;9	1.25	8;8	1.25	9;9										
	1.00	9;D	1.00	8;8	1.00	9;9										
	0.75	D;D	0.75	9;9	0.75	D;D										
	0.50	D;D	0.50	9;9	0.50	D;D										

	Antitoxin/Toxin Control															
	Assay 1		Assay 2		Assay 3		Assay 4		Assay 5		Assay 6		Assay 7		Assay 8	
Pre-dilution	1		1		1											
Lab 9 L^+ = 170	1.50	6;6	1.50	6;6	1.50	5;5										
	1.25	7;7	1.25	7;7	1.25	6;6										
	1.00	8;8	1.00	8;8	1.00	7;7										
	0.75	9;8	0.75	8;8	0.75	8;8										
	0.50	9;9	0.50	9;9	0.50	9;9										
Pre-dilution	16		8		8											
Lab 10 L^+ = 170	1.50	3;3	1.50	4;4	1.50	4;3										
	1.25	4;4	1.25	5;5	1.25	5;5										
	1.00	5;5	1.00	5;5	1.00	5;5										
	0.75	5;5	0.75	6;6	0.75	6;6										
	0.50	5;6	0.50	6;6	0.50	6;6										
	999	1;1	999	L;L	999	L;L										
Pre-dilution	8		8		8		8		8		8		8			
Lab 11 L^+ = 133.3	1.50	L;L	1.50	L;L	1.50	L;L	1.50	L;L	1.50	L;L	1.50	L;L	1.50	L;L		
	1.25	3;3	1.25	3;3	1.25	2;2	1.25	2;2	1.25	2;2	1.25	2;2	1.25	2;2		
	1.00	3;3	1.00	3;4	1.00	3;3	1.00	3;3	1.00	3;4	1.00	3;4	1.00	3;4		
	0.75	4;3	0.75	4;4	0.75	4;4	0.75	4;4	0.75	5;4	0.75	4;4	0.75	4;4		
	0.50	5;4	0.50	4;5	0.50	4;5	0.50	4;4	0.50	5;5	0.50	5;5	0.50	5;5		

Table F – MLD predictions in vivo from in vitro testing. Summary overview of endpoints

	TxA						TxB						TxC						TxD						TxE						TxF					
	Assay 1		Assay 2		Assay 3		Assay 1		Assay 2		Assay 3		Assay 1		Assay 2		Assay 3		Assay 1		Assay 2		Assay 3		Assay 1		Assay 2		Assay 3		Assay 1		Assay 2		Assay 3	
Pre-dil:	50		50		50		50		50		50		3		3		3		10		10		10		50		50		50		50		50		50	
Lab 2	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D	1	D;L	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D
	3	L;L	3	L;L	3	D;D	3	D;D	3	D;D	3	L;L	3	L;L	3	L;L	3	L;L	3	D;D	3	L;L	3	L;L	3	D;D	3	L;D	3	D;D	3	D;D	3	L;L	3	D;D
	9	L;L	9	L;L	9	L;L	9	L;L	9	L;L	9	L;L	9	L;L	9	L;L	9	L;L	9	L;L	9	L;L	9	L;L	9	L;L	9	L;L	9	L;L	9	L;L	9	L;L	9	L;L
	27	L;L	27	L;L	27	L;L	27	L;L	27	L;L	27	L;L	27	L;L	27	L;L	27	L;L	27	L;L	27	L;L	27	L;L	27	L;L	27	L;L	27	L;L	27	L;L	27	L;L	27	L;L
	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L
Pre-dil:	50		100		100		100		100		200		8		6		8		8		10		10		100		100		200		100		100		200	
Lab 3	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D
	3	D;D	3	D;D	3	D;D	3	D;D	3	D;D	3	L;L	3	D;D	3	D;D	3	D;D	3	D;D	3	D;D	3	D;D	3	D;D	3	D;D	3	L;D	3	D;D	3	D;D	3	D;D
	9	L;L	9	L;L	9	L;L	9	L;L	9	L;L	9	L;L	9	L;L	9	L;L	9	L;L	9	L;D	9	D;D	9	L;L	9	L;L	9	D;D	9	L;L	9	L;L	9	D;L	9	L;L
	27	L;L	27	L;L	27	L;L	27	L;L	27	L;L	27	X;X	27	L;L	27	L;L	27	L;L	27	L;L	27	L;L	27	L;L	27	L;L	27	L;L	27	L;L	27	L;L	27	L;L	27	L;L
	81	L;L	81	L;L	81	L;L	81	L;X	81	L;L	81	X;L	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L
Pre-dil:	4		4		4		4		4		4		1		1		1		1		1		1		4		4		4		4		4		4	
Lab 4	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D	1	L;L	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D
	3	D;D	3	D;D	3	D;D	3	D;D	3	L;L	3	D;D	3	L;L	3	L;L	3	L;L	3	D;D	3	D;D	3	D;D	3	D;D	3	D;D	3	D;D	3	D;D	3	D;D	3	D;D
	9	L;L	9	L;L	9	L;L	9	L;L	9	L;L	9	L;L	9	L;L	9	L;L	9	L;L	9	L;L	9	D;D	9	L;L	9	D;D	9	D;D	9	D;D	9	D;D	9	D;D	9	D;D
	27	L;L	27	L;L	27	L;L	27	L;L	27	L;L	27	L;L	27	L;L	27	L;L	27	L;L	27	L;L	27	L;L	27	L;L	27	L;L	27	L;L	27	L;L	27	L;L	27	L;L	27	L;L
	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L
Pre-dil:	10		10		10		5		5		5		1		1		1		3		3		3		5		5		5		5		5		5	
Lab 5	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D	1	L;D	1	D;L	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D
	3	D;D	3	D;D	3	D;D	3	D;D	3	D;D	3	D;D	3	L;L	3	L;L	3	L;L	3	D;D	3	D;D	3	D;D	3	D;D	3	D;D	3	D;D	3	D;D	3	D;D	3	D;D
	9	L;L	9	L;L	9	D;D	9	L;L	9	L;L	9	L;L	9	L;L	9	L;L	9	L;L	9	L;L	9	L;D	9	D;D	9	D;D	9	D;D	9	D;D	9	D;D	9	D;D	9	D;D
	27	L;L	27	L;L	27	L;L	27	L;L	27	L;L	27	L;L	27	L;L	27	L;L	27	L;L	27	L;L	27	L;L	27	L;L	27	L;L	27	L;L	27	D;D	27	D;D	27	D;D	27	D;D
	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L
Pre-dil:	28		28		28		17		17		17		3.3		3.3		3.3		2		2		2		17		17		17		17		17		17	
Lab 6	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D	1	D;D
	3	D;D	3	D;D	3	D;D	3	D;D	3	D;D	3	D;D	3	D;D	3	X;L	3	L;L	3	D;D	3	D;D	3	D;D	3	D;D	3	D;D	3	D;D	3	D;D	3	D;D	3	D;D
	9	D;D	9	L;L	9	L;L	9	D;L	9	L;L	9	L;L	9	L;L	9	L;L	9	L;L	9	D;D	9	D;D	9	D;D	9	D;D	9	D;D	9	D;D	9	D;D	9	D;D	9	D;D
	27	L;L	27	L;L	27	L;L	27	L;L	27	L;L	27	L;L	27	L;L	27	L;L	27	L;L	27	D;D	27	D;D	27	D;L	27	D;D	27	L;L	27	L;L	27	D;D	27	L;L	27	D;L
	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L	81	L;L