THE EUROPEAN DIRECTORATE FOR THE QUALITY OF MEDICINES & HEALTHCARE (EDQM)

Webinar on BINACLE Assay for Tetanus Neurotoxin: Outcomes of Project BSP136

12 November 2024

BINACLE Assay for Tetanus Neurotoxin: Outcomes of Project BSP136

EDQM & the Biological Standardisation Programme (BSP)

Catherine Milne, EDQM, Council of Europe

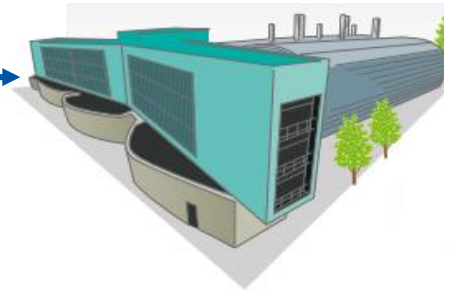
The EDQM, an entity within the Council of Europe

COUNCIL OF EUROPE

- Founded in **1949**
- **Intergovernmental** organisation, Strasbourg
- **46** Member States
- More than **700 Million** Citizens

COMMITTEE OF MINISTERS | PARLIAMENTARY ASSEMBLY | CONGRESS OF LOCAL AND REGIONAL AUTHORITIES | EUROPEAN COURT OF HUMAN RIGHTS | COMMISSIONER OF HUMAN RIGHTS | CONFERENCE OF INGOS

The **European Directorate for the Quality of Medicines & HealthCare** (EDQM)



edqm
European Directorate for the Quality of Medicines & HealthCare | Direction européenne de la qualité du médicament & soins de santé

COUNCIL OF EUROPE
CONSEIL DE L'EUROPE

- Founded in 1964
- Work in the framework of a **Partial Agreement, 39 Members & the EU**
- Ensures the availability of and access to good and safe quality medicines, Substances of Human Origin (SoHO) and consumer health products

EDQM 60 1964 - 2024 | COUNCIL OF EUROPE 75 1949 - 2024 CONSEIL DE L'EUROPE

EDQM activities in the area of medicinal products

European Pharmacopoeia: reference standards & methods

Binding in the **39** signatory states of the Ph. Eur, Convention and used as a reference worldwide; **33** observers from all continents

- More than **2 800 documentary standards** for the quality control of medicines
  - Cover the whole manufacturing process (e.g. excipients, medicinal products)
  - All stages of the life cycle of a medicine from development through to production and market surveillance
  - Methods verified & standardised

- **About 3000 reference standards shipped to 132 countries**

European Pharmacopeia Commission - treaty-based body - and its expert groups

Biological Standardisation Steering Committee

European Convention (ETS 123) for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes; and subsequently EU Dir 86/609 and its replacement EU Dir. 2010/63

PUBLIC HEALTH IMPACT

- **Ensure equivalent quality and safety of medicinal products throughout Europe and facilitate their free movement in Europe and beyond for all citizens**

EDQM Biological Standardisation Programme (BSP)

- Exists since 1991
- Contract with EU since 1994 - coincides with the creation of the OMCL network
- Sponsors
  - Council of Europe /EDQM
  - EU Commission

Funded
by the European Union
and the Council of Europe

EUROPEAN UNION

COUNCIL OF EUROPE

CONSEIL DE L'EUROPE

Implemented
by the Council of Europe

Goals of the EDQM BSP

Support Quality Control of Biologicals

- Establishment of Ph. Eur. standards (BRP/CRS) & reference reagents (BRR) – hereafter referred together as Reference Substances (RS)
- Standardisation of methods for Ph. Eur. for the quality control of biologicals
- Application of 3R concept (refine, reduce, replace) to Ph. Eur. methods
- International harmonisation: collaboration with WHO, OIE/WOAH and national authorities

Scope

- **Biotech products** (Group 6, MAB, P4BIO)
  (hormones, cytokines, anticoagulants (heparins), mAbs…)
- **Blood products, contaminants** (Group 6B)
  (immunoglobulins, coagulation factors…)
- **Vaccines, sera for human use** (Group 15)
- **Vaccines, sera for veterinary use** (Group 15V)
- **Miscellaneous** (specific working groups)
  (allergens, endotoxins, mycoplasma…)

How Does the EDQM BSP Help?

- **Provides a 'neutral' independent space for exchange**
  (anonymised participants /samples, central calculation)

  For:
  - Independent laboratories from national authorities e.g. OMCLs, NCLs
  - Laboratories from different manufacturers
  - Qualified academic/other labs
  - National authorities and experts

- **Uses large scale collaborative studies to:**
  - Demonstrate the general applicability and recommended use of methods already validated in a 'local/small scale' context
  - Establish commonly assigned values for Ph. Eur. reference standards (in IU where relevant)
- **Provides a key link between practical work of laboratories and the Ph. Eur.**
  - **Successful methods and standards are included in the Ph. Eur. and have a legal standing!**
- **Focus on the European market with an eye on global participation and acceptance** (common reference material and method recognition where possible)

Organisation

Steering Committee

Includes:

- Chairs of Ph. Eur. biological groups in the relevant fields
- Interested parties' representatives
  - European Medicines Agency (EMA); BWP, IWP
  - EU Commission
- Co-opted experts (human and vet)
- Representative of the EDQM Director
- Observer from World Health Organisation (WHO)

Project Leaders

- Nominated technical experts for a given study, bound by confidentiality agreements

EDQM:

- Technical secretariat; coordination & management of projects

Key Points for Study Proposals

Proposals

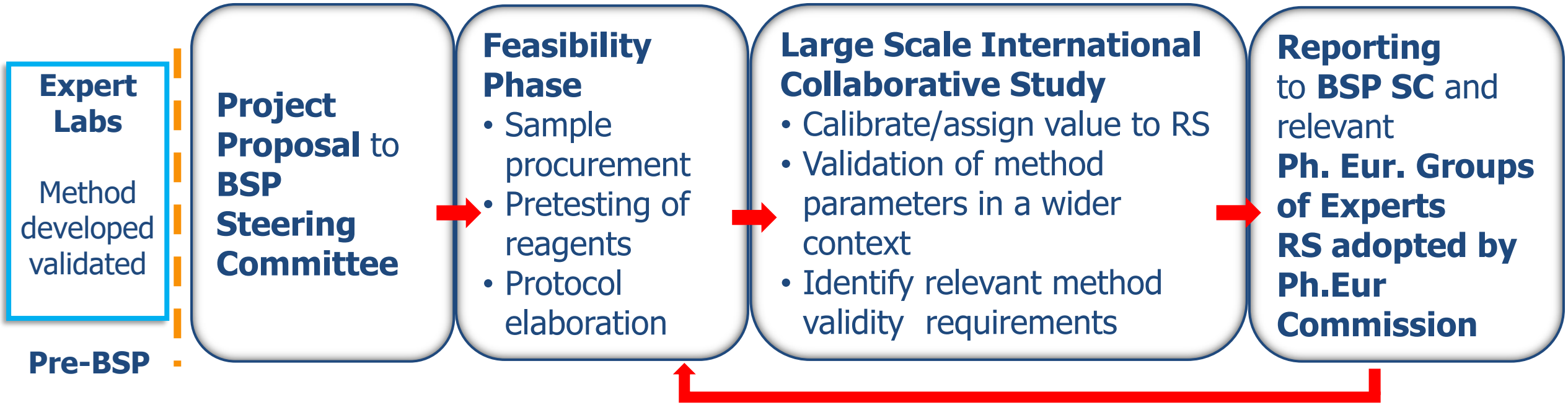
For new method (from Expert Group, OMCL, manufacturer…)

- ✓ Relevant to the Ph. Eur.
- ✓ Mature method with validation data (no research/development required)
- ✓ Not product specific
- ✓ No proprietary method/reagents
- ✓ 3R conscious
- ✓ External expert available as Project Leader

For RS

- ✓ Based on stock needs or new proposals
- ✓ For use in conjunction with Ph. Eur. methods
- ✓ Calibrate in IU when available/relevant
- ✓ If possible run jointly with WHO, and/or other regional/national standardisation bodies e.g. US-FDA/USP to established harmonised/common reference standards

BSP General Method of Work



Post-BSP: Dissemination of the study outcomes (publication*, conferences)
Method implementation by manufacturers and OMCLs
Regulatory acceptance (e.g. Ph. Eur. texts / acceptance by CA)

*PharmeuropaBio & Scientific Notes and other

Focus: The Collaborative Study

- Aims at
  - Determining the transferability of a method and general applicability with recommendations for validity criteria where relevant
  - Confirming the suitability of and calibrating / assigning a unitage to a candidate RS
- Includes
  - Study design in collaboration with a statistician
  - A pool of international participants : OMCLs, manufacturers, authorities

    variable number of laboratories depending on methods
    (with a view to adequate data sets for robust statistical evaluation)
  - a common protocol, calibrants (IS/BRP), reagents (as needed) & reporting sheets
  - a central analysis of the datasets – anonymised data
  - a review of the study report at successive levels:
    participants, relevant group of experts, BSP Steering Committee, Ph. Eur. Commission
  - a publication

Post-Collaborative Study Reporting Phase

Reporting Phase *with manufacturers (primarily)*

- **Optional phase based on specific need and study outcome**
  e.g. new reference standard, new method
- Collect and analyse data from routine production lots
  - Provides additional input on suitability
  - Helps to determine criteria for inclusion in Ph. Eur., specification setting

Dissemination of Results

BSP study reports are systematically published in the EDQM on-line publication

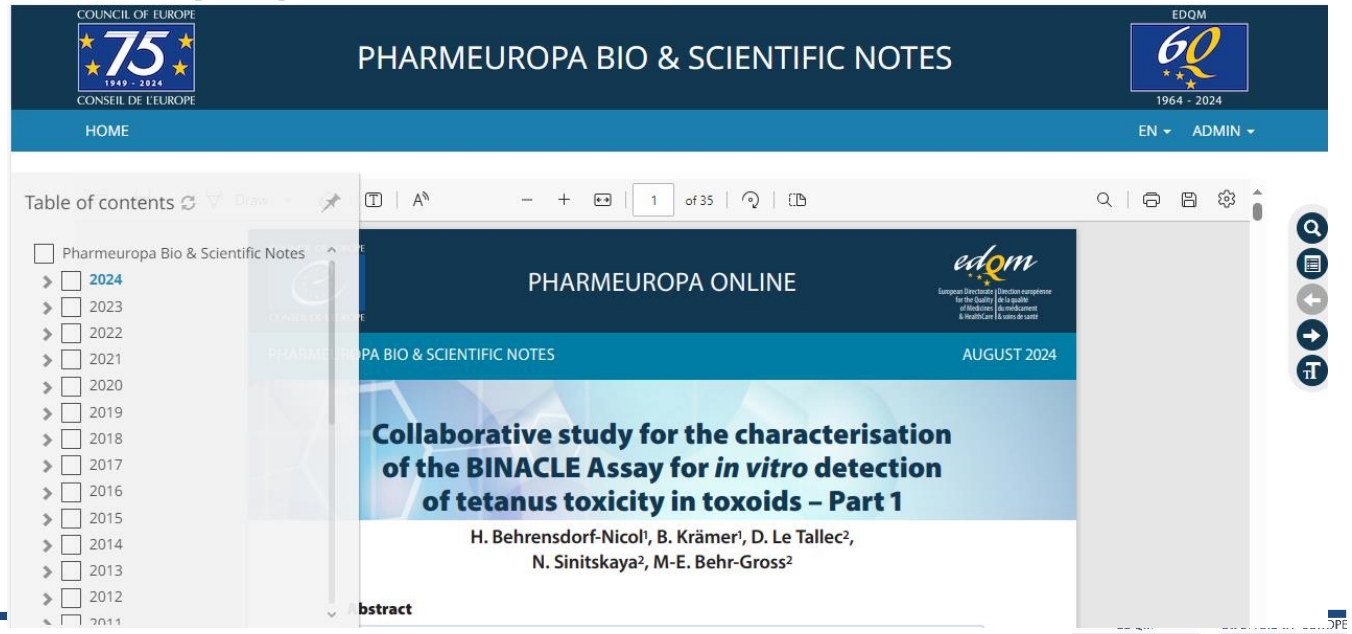
Pharmeuropa Bio & Scientific Notes

Studies may, in certain cases, also be published in other peer reviewed scientific journals

In some cases (e.g. new methods, issue of global interest) an interactive event is organised by EDQM e.g. webinar, symposium

FREE ONLINE ACCESS

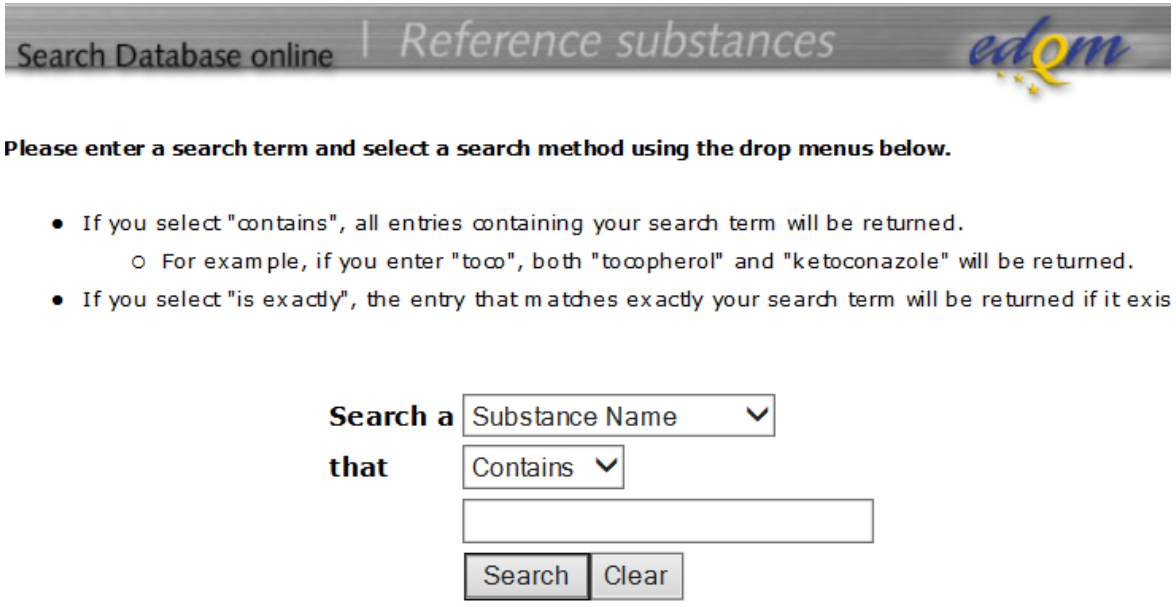
https://pbiosn.edqm.eu/home



Accessibility of RS, Leaflets, Batch Validity Statements

BRPs/BRRs/CRS are available via the EDQM online catalogue

https://crs.edqm.eu/

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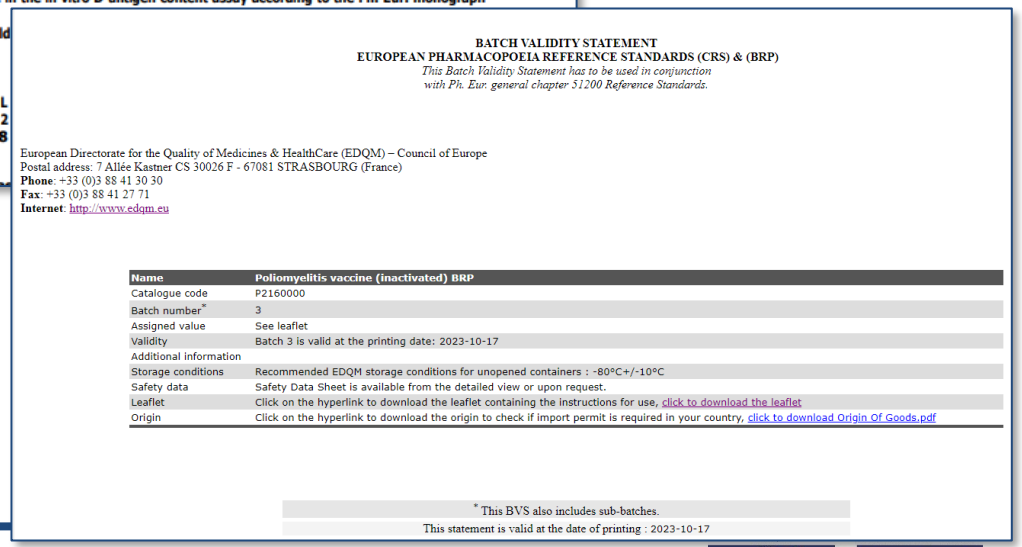
For any questions: www.edqm.eu (HelpDesk)

Information Leaflet Ph. Eur. Reference Standard
POLIOMYELITIS VACCINE (INACTIVATED) BRP batch 3

1. **Identification**
Catalogue code: P2160000
2. **Scientific Information**
2.1 Intended use
This leaflet supplements the currently valid European Pharmacopoeia monograph(s) and/or general chapter(s) describing the suitable use of this Reference Standard.
Further Information about the Reference Standard is available in the on-line catalogue currently at http://crs.edqm.eu (such as, batch validity statement and safety data statement).
2.2 Instructions for use
- The BRP is for use in the in vitro D-antigen content assay according to the Ph. Eur. monograph *0214*
- The material should

Content
The BRP consists of 0.6 mL
(Type 1 (Mahoney), Type 2
320- 78- 288


BATCH VALIDITY STATEMENT
EUROPEAN PHARMACOPOEIA REFERENCE STANDARDS (CRS) & (BRP)
This Batch Validity Statement has to be used in conjunction with Ph. Eur. general chapter 51200 Reference Standards.

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Phone: +33 (0)3 88 41 30 30
Fax: +33 (0)3 88 41 27 71
Internet: http://www.edqm.eu

Name	Poliomyelitis vaccine (inactivated) BRP
Catalogue code	P2160000
Batch number*	3
Assigned value	See leaflet
Validity	Batch 3 is valid at the printing date: 2023-10-17
Additional information	
Storage conditions	Recommended EDQM storage conditions for unopened containers : -80°C+/-10°C
Safety data	Safety Data Sheet is available from the detailed view or upon request.
Leaflet	Click on the hyperlink to download the leaflet containing the instructions for use, click to download the leaflet
Origin	Click on the hyperlink to download the origin to check if import permit is required in your country, click to download Origin Of Goods.pdf

*This BVS also includes sub-batches.
This statement is valid at the date of printing : 2023-10-17

Name, Batch #
Code
Intended Use
Instructions for use
Etc.

BVS: No pre-determined shelf-life
RS are monitored through their lifetime for suitability

BSP: A Rich History and a Bright Future

Over 30 years of experience with more than 180 BSP projects

~150 projects on BRP/CRS/BRR establishment: New and replacement batches of >70 different RS

> 40 projects related to methods: ~ 30 related to 3Rs - to refine, reduce, replace animal use

BSP contributes to the Ph. Eur.

- Large scale studies of methods to promote validation/implementation (particularly 3Rs)
- BRP/CRS/BRR that support the application of Ph. Eur. methods

With a view to

- The standardisation of biological medicines
- The protection of public health

Explore your opportunities to contribute to ongoing and new projects at

https://www.edqm.eu/en/bsp-work-programme

Many Thanks to All Supporters

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International Collaborators

EDQM BSP Team

EDQM Statisticians

..and all EDQM internal collaborators

Thank you for your attention

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BINACLE Assay for Tetanus Neurotoxin: Outcomes of Project BSP136

Session I: The BINACLE and BSP136

Moderator: Laurent Mallet,
EDQM, Council of Europe

The BINACLE (binding and cleavage) assay for *in vitro* activity determination of tetanus neurotoxin

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Bundesinstitut für Impfstoffe und biomedizinische Arzneimittel
Federal Institute for Vaccines and Biomedicines

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Paul-Ehrlich-Institut

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Health is at the heart of our work

- Federal higher authority based in Langen near Frankfurt am Main – in the heart of Germany and Europe.
- We contribute to the
  - **QUALITY, SAFETY** and **EFFICACY** as well as
  - **the AVAILABILITY**
  of vaccines and biomedicines.
- We test and evaluate vaccines and biomedicines, approve clinical trials in Germany and grant marketing authorisations.
- Our research focuses on model drugs and method development.
- ZEPAI represents our responsibility for planning and implementing pandemic preparedness and pandemic response measures with pandemic vaccines and therapeutics.
- Our expertise allows us to support groups such as medicines developers and manufacturers by providing regulatory scientific advice along the entire drug life cycle.
- We collect and evaluate incidents pertaining to certain in vitro diagnostic medical devices (e.g. CoV-2 rapid antigen tests) and approve performance studies.

TETANUS NEUROTOXIN AND THE BINACLE ASSAY

Tetanus neurotoxin

Tetanus neurotoxin (TeNT) produced by *Clostridium tetani*

- Targets inhibitory interneurons → **Muscle spasms**, asphyxiation
- Extremely **potent** (lethal dose for humans + many animals: **~1-10 ng/kg** body weight)

Chemically inactivated TeNT (**tetanus toxoid**) is used as **vaccine**

- Each bulk must be tested for absence of active TeNT
- Due to high toxicity: **Reliable method for toxin detection** needed

European Pharmacopoeia: Test for **"Absence of tetanus toxin"** (veterinary + human vaccines)

- Toxoid injected into 5 **guinea pigs,** 21 days observation phase
- No animal should show tetanus symptoms
- No generally accepted **alternative method** to date

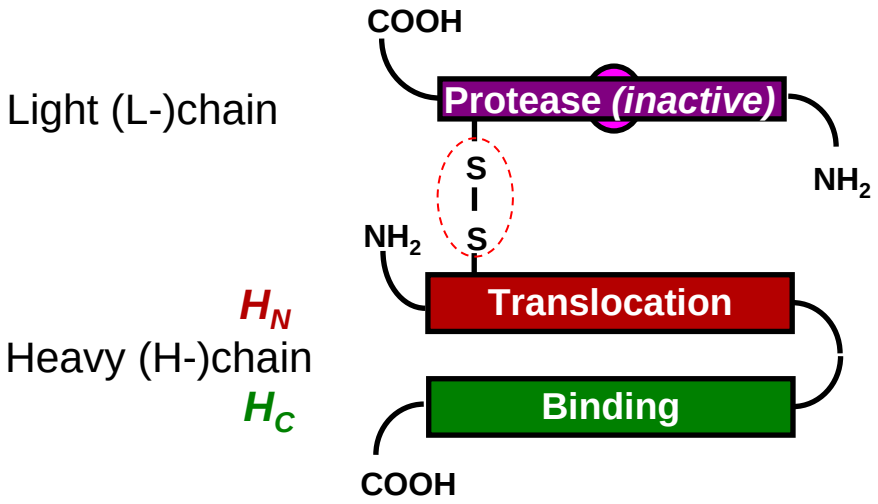
Reasons for developing *in vitro* methods

Disadvantages of *in vivo* test:

- Ethical concerns
- High variability + low precision
- Guinea pig test was introduced decades ago
  → poorly standardised, not properly validated (e.g. detection limit unknown)
- Long duration (3 weeks observation phase)
- Expensive (animal facilities)

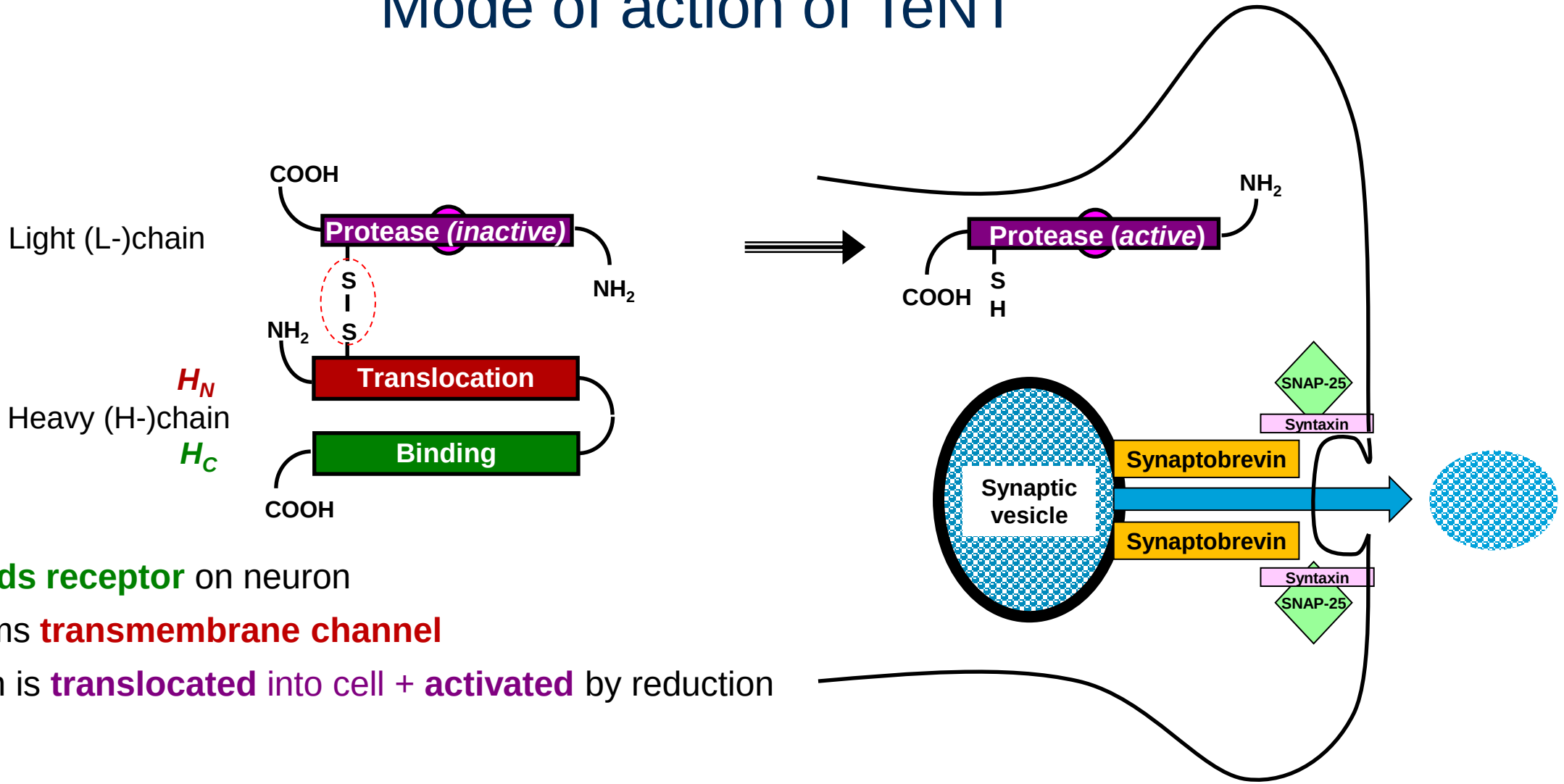
→ Replacement by *in vitro* method preferable

Mode of action of TeNT



1. H_C **binds receptor** on neuron
2. H_N forms **transmembrane channel**

Mode of action of TeNT



1. H_C **binds receptor** on neuron
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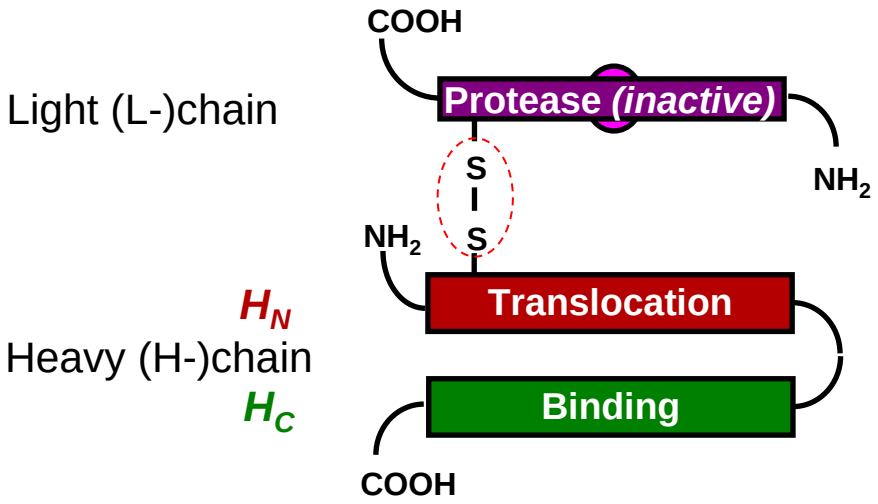
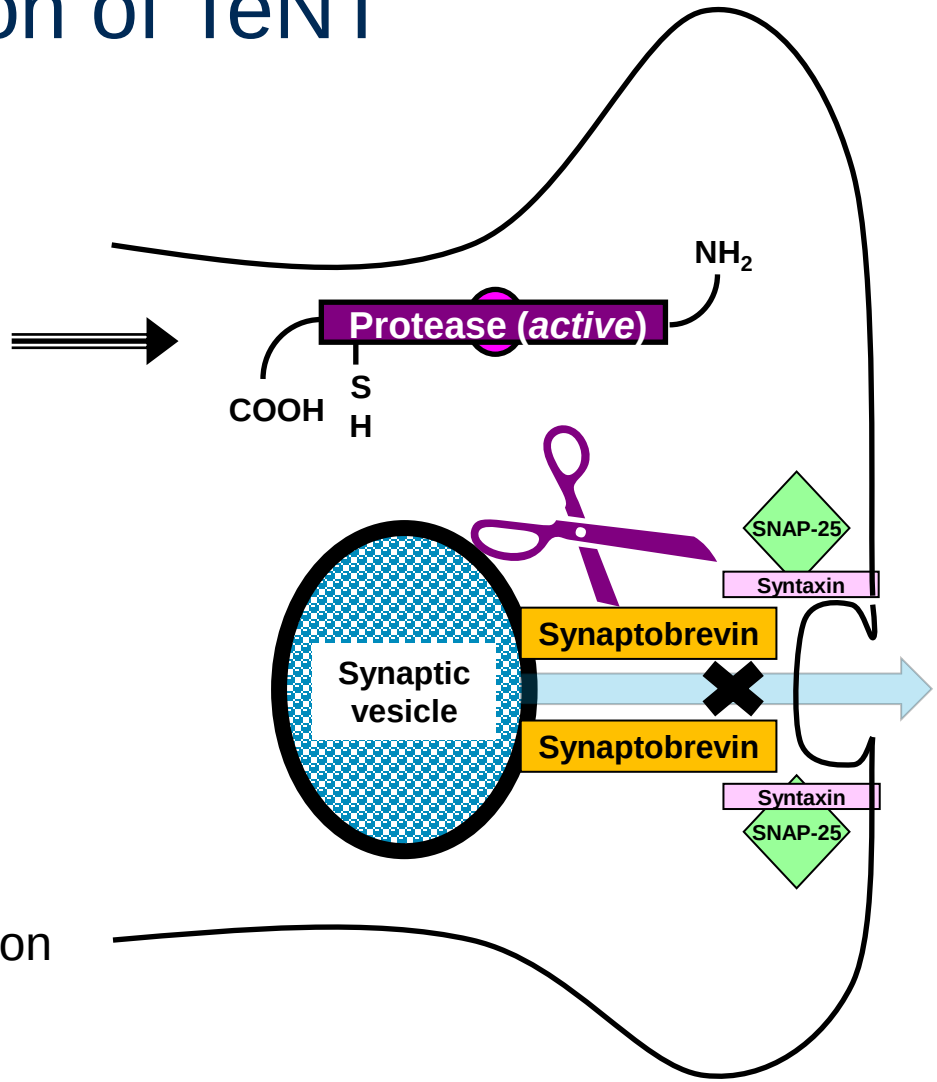
3. L-chain is **translocated** into cell + **activated** by reduction

Mode of action of TeNT

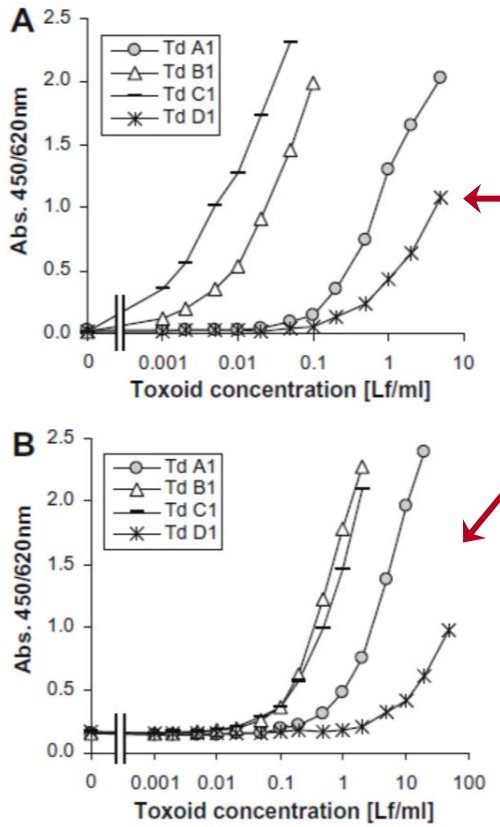




1. H_C **binds receptor** on neuron
2. H_N forms **transmembrane channel**
3. L-chain is **translocated** into cell + **activated** by reduction
4. L-chain **cleaves synaptobrevin**

⇨ **Neurotransmitter release** is blocked
⇨ **Severe spasms**

Single assays (binding assay / endopeptidase assay)



[Figure from: Behrensdorf-Nicol HA, Bonifas U, Kegel B, Silberbach K, Krämer B, Weißer K (2010) Toxicology In Vitro 24:988-994]

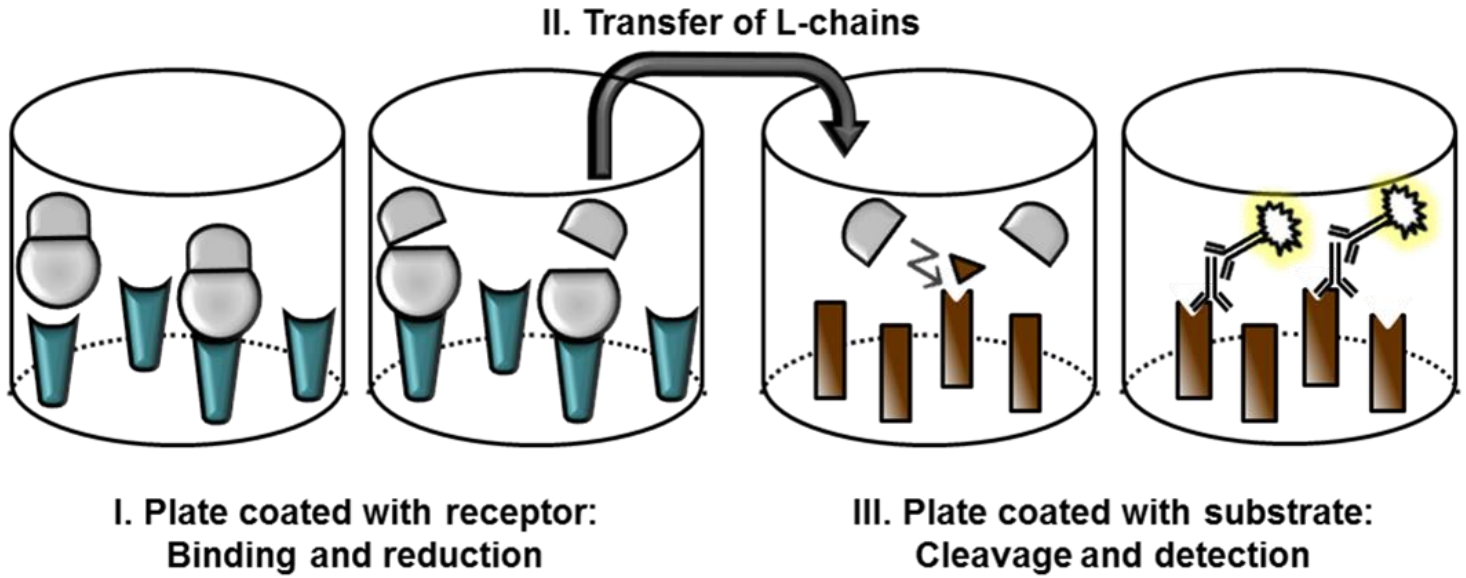
Toxoids (various manufacturers) tested in

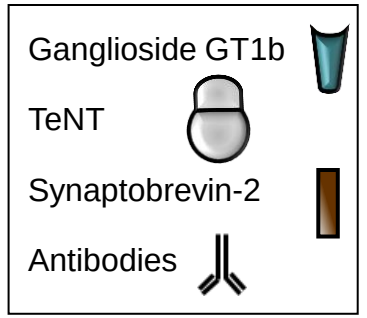
(A) **receptor-binding assay**
(B) assay for **synaptobrevin-cleaving** activity

- All toxoids: **High signals** (already at concentrations <10 Lf/ml)
- Signals do not correspond to *in vivo* toxicity (all toxoids had passed the animal test)

→ Single assays: **No reliable discrimination between active toxin and inactivated toxoid molecules**

BINACLE assay principle



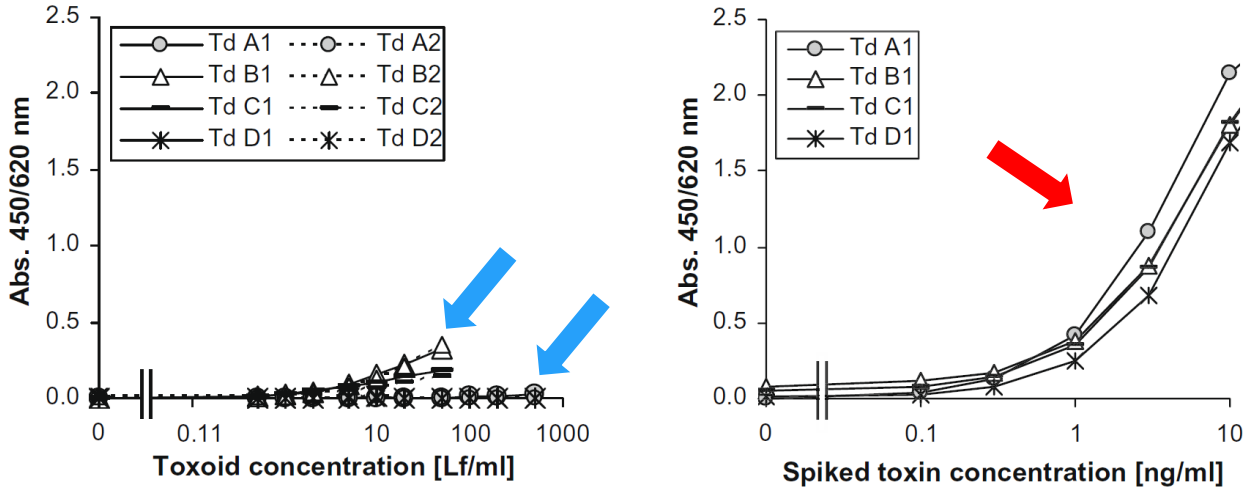


[Figure adapted from: Behrensdorf-Nicol H, Weisser K, Krämer B (2015), ALTEX 32:41-46]

BINACLE (binding and cleavage) assay for ***in vitro*** **activity determination**

- mimics key steps of TeNT mode of action**: Receptor binding** + **synaptobrevin cleavage**
- detects **active TeNT molecules** based on several characteristic features:
  - functional binding domain (H-chain) + functional protease domain (L-chain)
  - both chains must be separable by reduction

BINACLE assay allows TeNT detection in toxoids



[Figure from: Behrensdorf-Nicol HA, Bonifas U, Kegel B, Silberbach K, Krämer B, Weißer K (2010) Toxicology In Vitro 24:988-994]

Non-spiked toxoids:

- For some toxoids, BINACLE signals did not exceed blank value even when tested at high concentration (500 Lf/ml)
- Toxoids from some sources induce elevated background signals when tested in high concentrations

Toxoids spiked with TeNT:

- Clear dose-response-relationship
- Sensitive TeNT detection

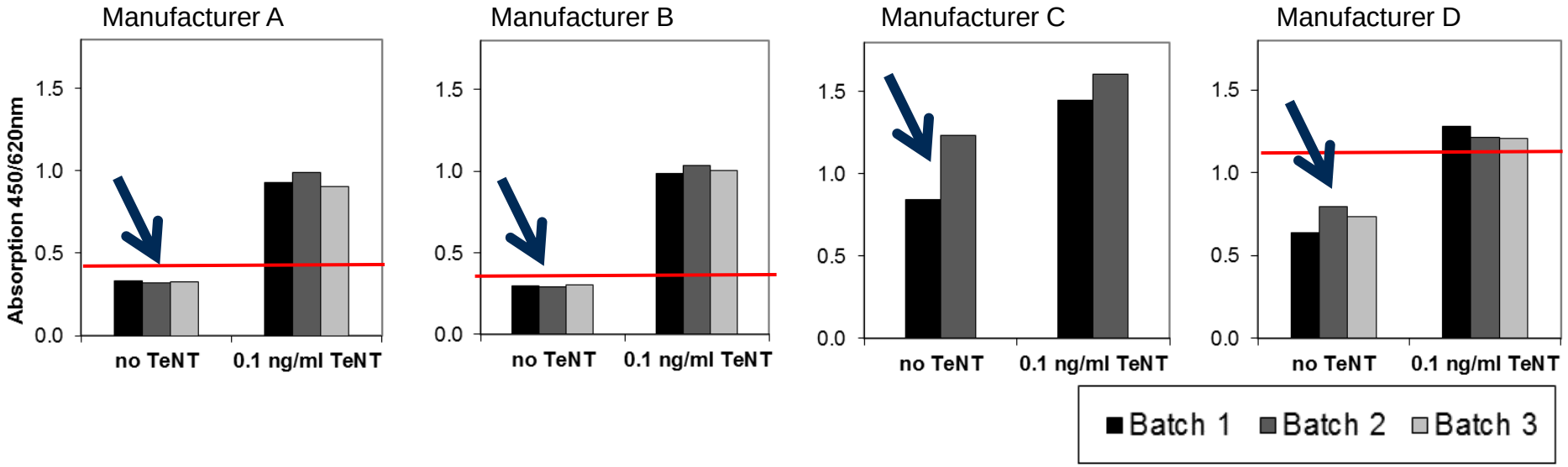
→ **BINACLE assay:** Strongly improved **discrimination between active toxin and inactivated toxoid molecules** compared to single assays

(**Note:** Toxoids from some sources induce elevated background signals, such toxoids may be less suitable for BINACLE testing)

IN-HOUSE CHARACTERISATION AND TRANSFERABILITY STUDY

In-house characterisation of BINACLE assay

Toxoid batches from 4 vaccine manufacturers:



[Figure modified from: Behrensdorf-Nicol HA, Bonifas U, Hanschmann KM, Krämer B, Weißer K (2013) Vaccine 31:6247-6253]

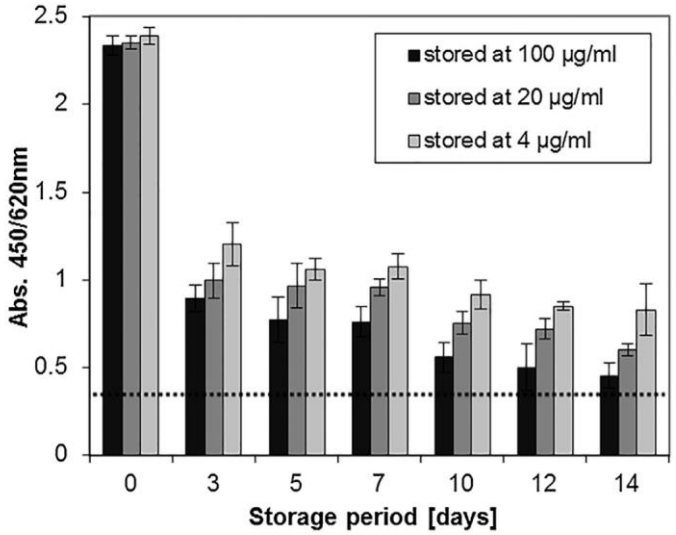
Batch-to-batch variability of toxoids from same manufacturer: usually low

Manufacturer-specific cut-off (= mean of non-spiked samples + 3-fold standard deviation) can be used to discriminate between TeNT-spiked and TeNT-negative samples

→ 0.1 ng/ml TeNT (≈ estimated detection limit of animal test) detected in most cases

Toxoids from some manufacturers induce high signals that are not related to toxicity, thus toxin detection by BINACLE may not be possible for all toxoids

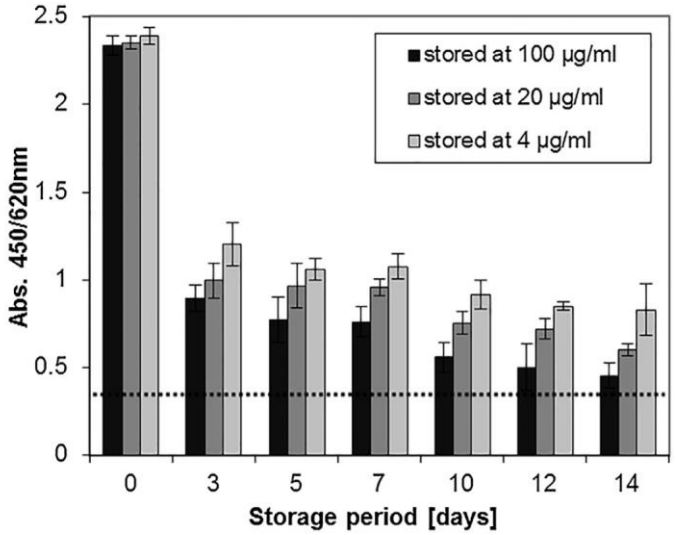
Detection of changes induced by storage at 37°C



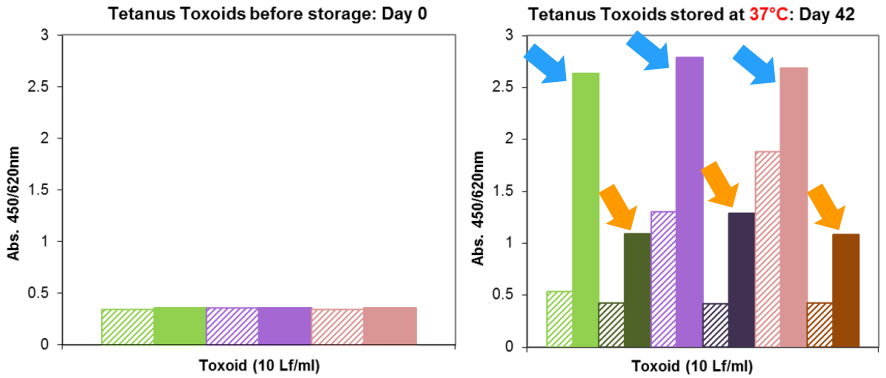
[Figure from: Behrensdorf-Nicol HA, Krämer B (2019) Vaccine 37:1721-1724]

BINACLE detects decrease in TeNT activity resulting from storage of toxin solutions at **elevated temperature** (37°C)

Detection of changes induced by storage at 37°C



[Figure from: Behrensdorf-Nicol HA, Krämer B (2019) Vaccine 37:1721-1724]



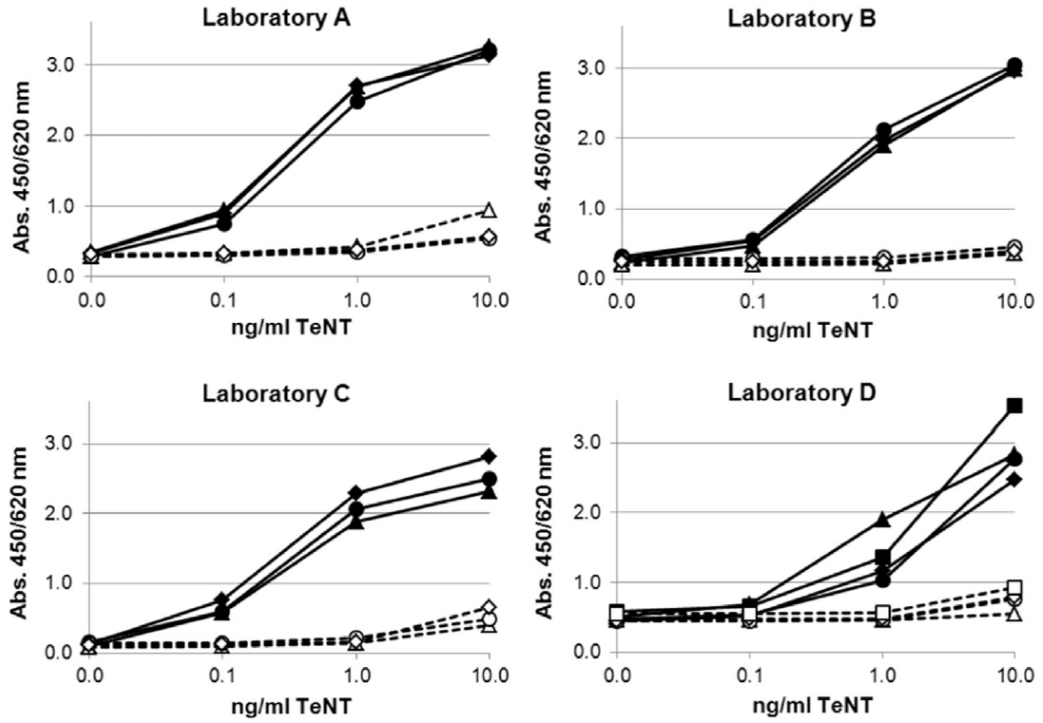
BINACLE detects decrease in TeNT activity resulting from storage of toxin solutions at **elevated temperature** (37°C)

BINACLE data indicate that **storage at 37°C** can also induce **changes in toxoids:**

- **Elevated signals** after storage for 42 d
  (more pronounced for toxoids diluted to 20 Lf/ml than for stock solutions)
- Signals did not correspond to actual toxicity (toxoids had passed animal test for "absence of toxin and irreversibility of toxoid")

Issue was not **pursued further, as test on 37°C-stored toxoid is no longer relevant** (due to deletion of irreversibility test from Ph. Eur.)

Results of transferability study



[Figure from: Behrensdorf-Nicol HA, Bonifas U, Isbrucker R, Ottiger H, Tierney R, Hanschmann KM, Weisser K, Krämer B (2014) Biologicals 42:199-204]

Study design:

- 4 international laboratories
- 13 BINACLE tests performed in total
  (Each curve shows results from one assay, dotted lines: controls without receptor)
- Test samples: TeNT-spiked toxoid

Results:

- All participants successfully performed the BINACLE assays
- Toxin concentration-dependent signal increase observed in all tests
- 0.1 ng/ml TeNT spiked into toxoids: Detected in most tests

INITIATION OF COLLABORATIVE STUDY BSP136

Conclusions from in-house and transferability data

The previous studies had indicated that:

- BINACLE assay is capable of **detecting spiked TeNT in relevant toxoids**
  (applicability may differ between toxoids from different sources)
- **LOD** may be in a **similar range** as the estimated LOD of the **animal test**
- **Lab-to-lab transferability** was shown in small-scale study

→ BINACLE might represent a **suitable *in vitro* method** for **toxoid safety testing**

→ **Next step:** Study involving a larger number of laboratories to achieve a characterisation of the method on a broader data basis

Collaborative study BSP136

International collaborative study BSP136 for tetanus BINACLE assay

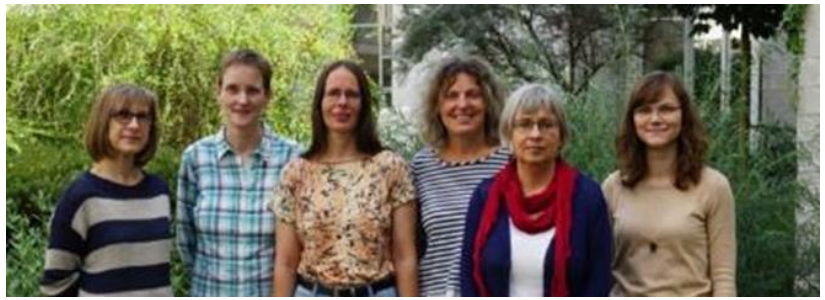
- organised by EDQM
- in the context of the Biological Standardisation Programme (BSP)

→**Aim**: Characterise **applicability of BINACLE assay** for ***in vitro*** **toxicity testing of tetanus toxoids**

Acknowledgements

Project team at Paul-Ehrlich-Institut:

Ursula Bonifas, Jolanta Klimek, Beate Krämer, Birgit Kegel, Karin Weißer, Emina Wild, Kay-Martin Hanschmann



EDQM (European Directorate for the Quality of Medicines & HealthCare, Strasbourg, France)**:**

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AnimalfreeResearch

DFG Deutsche Forschungsgemeinschaft German Research Foundation

THANK YOU VERY MUCH

FOR YOUR ATTENTION

EDQM webinar

Review and harmonisation of **toxicity testing requirements** in Ph. Eur. Tetanus vaccine monographs: **3Rs achievements**

12 November 2024

Gwenaël Ciréfice & Catherine Lebrun, EDQM

A stepwise review of Tetanus vaccine monographs

1. Reassessing the *Test for irreversibility of toxoid*
2. Rationalising toxicity testing requirements for Tetanus vaccines (deletion of the *Test for specific toxicity*)
3. Harmonisation of Tetanus vaccine monographs (human and vet use)

Reassessing the *Test for irreversibility of toxoid* in Tetanus vaccine monographs

- In 2015, a study using the BINACLE assay showed that the functional activity of Tetanus Neurotoxin rapidly decreased during storage at 37°C. This confirmed earlier reports regarding the stability of the TeNT over storage at 37°C, prompting a re-examination of the *Test for irreversibility* described in the monographs *Tetanus vaccine (adsorbed)* (0452) & *Tetanus vaccine for veterinary use* (0697), performed at 37°C.

- Data (published and in house data, survey with vaccine manufacturers) were collected and analysed. Analysis of these data led to the conclusion that **the toxin is not stable and loses neurotoxic activity** under the conditions of the storage test **at 37˚C**. In this context, toxoid samples cannot be expected to contain relevant amounts of active TeNT after 6 weeks of storage at 37°C.
- A **review of the whole design** of the test *Absence of toxin and irreversibility of toxoid* was carried out, including the test after storage at 5°C (used as control)

- A revision of monographs 0452 & 0697 was launched in 2018 to propose the deletion of the *Test for irreversibility (test after storage at 37˚C and control at 5°C).*

Rationalising toxicity testing requirements for Tetanus vaccines (deletion of the *Test for specific toxicity*)

- During the subsequent review of the monographs by Group 15 / Group 15V, it was noted that the *Test for Specific Toxicity / Test for Residual Toxicity*, which could be used for Final Bulk or Final Lot, may be redundant given that the more sensitive *Test for Absence of tetanus toxin* is performed on the bulk toxoid.
- The scope of the revision was thus **extended** in order include a **review of the *Test for Specific Toxicity / Test for Residual Toxicity*** as part of the same exercise
- A survey with manufacturers was also launched to collect data

Harmonisation of Tetanus vaccine monographs (human and vet use)

- Different situation for human and veterinary vaccines but the same purified tetanus toxoid is used for the production of human and vet vaccines → **take advantage of this revision to harmonise both monographs**

Tetanus vaccine (adsorbed) (0452)

Ph. Eur. 6.0

01/2008:0452
corrected 6.0

TETANUS VACCINE (ADSORBED)

Vaccinum tetani adsorbatum

Irreversibility of Toxoid:
- **Storage at 37°C over 6 weeks**
- **Storage at 2-8°C over 6 weeks** (control)

PRODUCTION

GENERAL PROVISIONS

Specific toxicity. The production method is validated to demonstrate that the product, if tested, would comply with the following test: inject subcutaneously 5 times the single human dose stated on the label into ea
each weighing 250-350 g, that h
with any material that will inte
21 days of the injection any of
dies from tetanus, the vaccine
If more than 1 animal dies from non-specific causes, repeat the test once; if more than 1 animal dies in the second test, the vaccine does not comply with the test.

Absence of Toxin

Only bulk purified toxoid that complies with the following requirements may be used in the preparation of the final bulk vaccine.

Sterility (*2.6.1*). Carry out the test for sterility using 10 mL for each medium.

Absence of toxin and irreversibility of toxoid. Using the same buffer solution as for the final vaccine, without adsorbent, prepare a solution of bulk purified toxoid at the same concentration as in the final vaccine. Divide the dilution into 2 equal parts. Keep one of them at 5 ± 3 °C and the other at 37 °C for 6 weeks. Test both dilutions as described below. Use 15 guinea-pigs, each weighing 250-350 g and that have not previously been treated with any material that will interfere with the test. Inject subcutaneously into each of 5 guinea-pigs 5 mL of the dilution incubated at 5 ± 3 °C. Inject subcutaneously into each of 5 other guinea-pigs 5 mL of the dilution incubated at 37 °C. Inject subcutaneously into each of 5 guinea-pigs at least 500 Lf of the non-incubated bulk purified toxoid in a volume of 1 mL. The bulk purified toxoid complies with the test if during the 21 days following the injection no animal shows signs of or dies from tetanus. If more than 1 animal dies from non-specific causes, repeat the test; if more than 1 animal dies in the second test, the toxoid does not comply with the test.

Antigenic purity (*2.7.27*). Not less than 1000 Lf per milligram of protein nitrogen.

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Tetanus vaccine (adsorbed) (0452) *(cont'd)*

Ph. Eur. 6.0

TETANUS VACCINE (ADSORBED)

Vaccinum tetani adsorbatum

DEFINITION

Tetanus vaccine (adsorbed) is a preparation of tetanus formol toxoid with a mineral adsorbent. The formol toxoid is prepared from the toxin produced by the growth of *Clostridium tetani*.

PRODUCTION

GENERAL PROVISIONS

Specific toxicity. The production method is validated to demonstrate that the product, if tested, would comply with the following test: inject subcutaneously 5 times the single human dose stated on the label into each of 5 healthy guinea-pigs, each weighing 250-350 g, that have not previously been treated with any material that will interfere with the test. If within 21 days of the injection any of the animals shows signs of or dies from tetanus, the vaccine does not comply with the test. If more than 1 animal dies from non-specific causes, repeat the test once; if more than 1 animal dies in the second test, the vaccine does not comply with the test.

Only bulk purified toxoid that complies with the following requirements may be used in the preparation of the final bulk vaccine.

Sterility (*2.6.1*). Carry out the test for sterility using 10 mL for each medium.

Absence of toxin and irreversibility of toxoid. Using the same buffer solution as for the final vaccine, without adsorbent, prepare a solution of bulk purified toxoid at the same concentration as in the final vaccine. Divide the dilution into 2 equal parts. Keep one of them at 5 ± 3 °C and the other at 37 °C for 6 weeks. Test both dilutions as described below. Use 15 guinea-pigs, each weighing 250-350 g and that have not previously been treated with any material that will interfere with the test. Inject subcutaneously into each of 5 guinea-pigs 5 mL of the dilution incubated at 5 ± 3 °C. Inject subcutaneously into each of 5 other guinea-pigs 5 mL of the dilution incubated at 37 °C. Inject subcutaneously into each of 5 guinea-pigs at least 500 Lf of the non-incubated

Specific toxicity:
- **During product development, on finished product**

does not comply with the test.

Antigenic purity (*2.7.27*). Not less than 1000 Lf per milligram of protein nitrogen.

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Review of toxicity testing requirements for Tetanus vaccines

- ***Test for Absence of toxin*** **kept as is**
- ***Test for irreversibility*** of toxoid (test in guinea pigs) **deleted**
  - Rationale: Tetanus toxin was shown to lose neurotoxic activity under the conditions of the storage test at 37°C; Data confirming the stability of Tetanus toxoid
- ***Test for specific toxicity*** (process validation requirement, test in guinea pigs) **removed**
  - Rationale: Considered redundant because a more sensitive test for residual toxin is performed routinely on bulk purified toxoid. The emphasis is placed on the need to validate the detoxification process to demonstrate that the toxoid is stably detoxified

Tetanus vaccine (adsorbed) (0452) – Suppl. 10.3

European Pharmacopoeia 11.7

Home | 11th Edition | Archives

EDITION 11.0: French version corrected

SUPPLEMENT 10.3

Specific toxicity. The requirement to perform the test for specific toxicity on the product as part of validation of the production process was considered redundant because a more sensitive test for residual toxin is performed routinely on the bulk purified toxoid and the revised monograph emphasises the need to validate the detoxification process to demonstrate that the toxoid is stably detoxified.

Absence of toxin and irreversibility of toxoid. The test for irreversibility of the tetanus toxoid is no longer regarded as relevant, in view of data on the stability of the tetanus toxoid and the fact that the tetanus toxin was shown to lose neurotoxic activity under the conditions of the storage test at 37 °C. The test after storage at 5 °C, which was used as a control in the test for irreversibility, has also been removed. The more sensitive test for absence of toxin carried out on non-incubated purified toxoid has been retained.

EDITION 10.0: English version corrected

Knowledge Database

Search Database online | Knowledge Database

Detailed view of Tetanus vaccine (adsorbed).

Status	In use
Monograph Number	00452
English Name	Tetanus vaccine (adsorbed)
French Name	Vaccin tétanique adsorbé
Latin Name	Vaccinum tetani adsorbatum
Pinyin Name	
Chinese Name	
Pharmeuropa	31.2
Published in English Supplement	10.3
Published in French Supplement	11.0
On-going	Revision
State of work	2 - Pharmeuropa
Pharmeuropa	
Description	Introduction of a reference to BINACLE as an in vitro alternative to the test for Absence of tetanus toxin in guinea pigs.
Chromatogram	Not available
Additional information	Not available
History	View history
Interchangeable (ICH_Q4B)	NO
Pharmacopoeial harmonisation	NO
Reference standards	
Practical Information	Test(s) / Brand Name/Information
CEP	

Tetanus vaccine (adsorbed) (0452) – Suppl. 10.3

01/2021:0452

Current text

TETANUS VACCINE (ADSORBED)

Vaccinum tetani adsorbatum

DEFINITION

Tetanus vaccine (adsorbed) is a preparation of tetanus formol toxoid with a mineral adsorbent. The formol toxoid is prepared from the toxin produced by the growth of *Clostridium tetani*.

PRODUCTION

BULK PURIFIED TOXOID

For the production of tetanus toxin, from which toxoid is prepared, seed cultures are managed in a defined seed-lot system in which toxinogenicity is conserved and, where necessary, restored by deliberate reselection. A highly toxinogenic strain of *C. tetani* with known origin and history is grown in a suitable liquid medium. At the end of cultivation, the purity of each culture is tested and contaminated cultures are discarded. Toxin-containing culture medium is collected aseptically. The toxin content (Lf per millilitre) is checked (*2.7.27*) to monitor consistency of production. Single harvests may be pooled to prepare the bulk purified toxoid. The toxin is purified to remove components likely to cause adverse reactions in humans. The purified toxin is detoxified with formaldehyde. The detoxification process is validated to demonstrate its ability to consistently produce a toxoid that is immunogenic and stably detoxified, including at the concentration used in the final lot. Alternatively, purification may be carried out after detoxification.

Only bulk purified toxoid that complies with the following requirements may be used in the preparation of the final bulk vaccine.

Sterility (*2.6.1*). Carry out the test for sterility using 10 mL for each medium.

Absence of tetanus toxin. Inject subcutaneously 1 mL containing at least 500 Lf of bulk purified toxoid into each of 5 healthy guinea-pigs , each weighing 250-350 g, that have not previously been treated with any material that will interfere with the test. If within 21 days of the injection any of the animals shows signs of or dies from tetanus, the toxoid does not comply with the test. If more than 1 animal dies from non-specific causes, repeat the test once; if more than 1 animal dies in the second test, the toxoid does not comply with the test.

Antigenic purity (*2.7.27*). Not less than 1000 Lf per milligram of protein nitrogen.

FINAL BULK VACCINE

The final bulk vaccine is prepared by adsorption of a suitable quantity of bulk purified toxoid onto a mineral carrier such as hydrated aluminium phosphate or aluminium hydroxide; the resulting mixture is approximately isotonic with blood. Suitable antimicrobial preservatives may be added. Certain antimicrobial preservatives, particularly those of the phenolic type, adversely affect the antigenic activity and must not be used.

Only final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended amount.

Sterility (*2.6.1*). Carry out the test for sterility using 10 mL for each medium.

FINAL LOT

The final bulk vaccine is distributed aseptically into sterile, tamper-evident containers. The containers are closed so as to prevent contamination.

Only a final lot that is satisfactory with respect to each of the requirements given below under Identification, Tests and Assay may be released for use. Provided the test for antimicrobial preservative and the assay have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

Provided the free formaldehyde content has been determined on the bulk purified toxoid or on the final bulk and it has been shown that the content in the final lot will not exceed 0.2 g/L, the test for free formaldehyde may be omitted on the final lot.

IDENTIFICATION

Tetanus toxoid is identified by a suitable immunochemical method (*2.7.1*). The following method, applicable to certain vaccines, is given as an example. Dissolve in the vaccine to be examined sufficient *sodium citrate R* to give a 100 g/L solution. Maintain at 37 °C for about 16 h and centrifuge until a clear supernatant is obtained. The clear supernatant reacts with a suitable tetanus antitoxin, giving a precipitate.

TESTS

Aluminium (*2.5.13*): maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

Free formaldehyde (*2.4.18*): maximum 0.2 g/L.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not greater than 115 per cent of the quantity stated on the label.

Sterility (*2.6.1*). The vaccine complies with the test for sterility.

[...]

Comparison of tetanus vaccine monographs 0452 & 0697

Ph. Eur. 8.3

TETANUS VACCINE FOR VETERINARY USE (0697)

Version 04/2013:0697 corrected 8.3

2-3. *MANUFACTURER'S TESTS*

2-3-1. Absence of toxin and irreversibility of toxoid.

Carry out a test for reversion to toxicity on the detoxified harvest using 2 groups of 5 guinea-pigs, each weighing 350-450 g; if the vaccine is adsorbed, carry out the test with the shortest practical time interval before adsorption.

Prepare a dilution of the detoxified harvest so that the guinea-pigs each receive 10 times the amount of toxoid (measured in Lf units) that will be present in a dose of vaccine.

Divide the dilution into 2 equal parts. Keep 1 part at 5 ± 3°C and the other at 37°C for 6 weeks. Attribute each dilution to a separate group of guinea-pigs and inject into each guinea-pig the dilution attributed to its group.

Observe the animals at least daily for 21 days.

The toxoid complies with the test if no guinea-pig shows signs of disease or dies from causes attributable to the neurotoxin of *C. tetani*.

2-3-1. Absence of toxin and irreversibility of toxoid.

3. BATCH TESTS

3-3. Residual toxicity.

Administer 5 mL of the vaccine by the subcutaneous route, as 2 equal divided doses at separate sites, into each of 5 healthy guinea-pigs, each weighing 350-450 g, that have not previously been treated with any material that will interfere with the test.

The vaccine complies with the test if no animal shows notable signs of disease or dies from causes attributable to the vaccine.

If within 21 days of the injection any of the animals shows signs of or dies from tetanus, the vaccine does not comply with the test. If more than 1 animal dies from non-specific causes, repeat the test. If any animal dies in the 2nd test, the vaccine does not comply with the test.

3-3. Residual toxicity.

Ph. Eur. 6.0

TETANUS VACCINE adsorbed (0452)

Version 01/2008:0452 corrected 6.0

GENERAL PROVISIONS

Specific toxicity.

The production method is validated to demonstrate that the product, if tested, would comply with the following test: inject subcutaneously 5 times the single human dose stated on the label into each of 5 healthy guinea-pigs, each weighing 250-350 g, that have not previously been treated with any material that will interfere with the test. If within 21 days of the injection any of the animals shows signs of or dies from tetanus, the vaccine does not comply with the test. If more than 1 animal dies from nonspecific causes, repeat the test once; if more than 1 animal dies in the second test, the vaccine does not comply with the test.

Specific toxicity.

BULK PURIFIED TOXOID

Absence of toxin and irreversibility of toxoid.

Absence of toxin and irreversibility of toxoid.

Using the same buffer solution as for the final vaccine, without adsorbent, prepare a solution of bulk purified toxoid at the same concentration as in the final vaccine. Divide the dilution into 2 equal parts. Keep one of them at 5 ± 3 °C and the other at 37 °C for 6 weeks. Test both dilutions as described below. Use 15 guinea-pigs, each weighing 250-350 g and that have not previously been treated with any material that will interfere with the test. Inject subcutaneously into each of 5 guinea-pigs 5 mL of the dilution incubated at 5 ± 3 °C. Inject subcutaneously into each of 5 other guinea-pigs 5 mL of the dilution incubated at 37 °C. Inject subcutaneously into each of 5 guinea-pigs at least 500 Lf of the non-incubated bulk purified toxoid in a volume of 1 mL.

The bulk purified toxoid complies with the test if during the 21 days following the injection no animal shows signs of or dies from tetanus. If more than 1 animal dies from non-specific causes, repeat the test; if more than 1 animal dies in the second test, the toxoid does not comply with the test.

Tetanus vaccine for veterinary use (0697)

04/2013:0697
corrected 8.3

TETANUS VACCINE FOR VETERINARY USE

Vaccinum tetani ad usum veterinarium

1. DEFINITION

Tetanus vaccine for veterinary use is a preparation of the neurotoxin of *Clostridium tetani* inactivated to eliminate its toxicity while maintaining adequate immunogenic properties. The vaccine may be used to induce active and/or passive immunity.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

2-3. *MANUFACTURER'S TESTS*

2-3-1. **Absence of toxin and irreversibility of toxoid.** Carry out a test for reversion to toxicity on the detoxified harvest using 2 groups of 5 guinea-pigs, each weighing 350-450 g; if the vaccine is adsorbed, carry out the test with the shortest practical time interval before adsorption. Prepare a dilution of the detoxified harvest so that the guinea-pigs each receive 10 times the amount of toxoid (measured in Lf units) that will be present in a dose of vaccine. Divide the dilution into 2 equal parts. Keep 1 part at 5 ± 3 °C and the other at 37 °C for 6 weeks. Attribute each dilution to a separate group of guinea-pigs and inject into each guinea-pig the dilution attributed to its group. Observe the animals at least daily for 21 days. The toxoid complies with the test if no guinea-pig shows signs of disease or dies from causes attributable to the neurotoxin of *C. tetani*.

3. BATCH TESTS

3-1. **Identification**

If the nature of the adjuvant allows it, carry out test A. Otherwise carry out test B.

A. Dissolve in the vaccine sufficient *sodium citrate R* to give a 100 g/L solution. Maintain the solution at 37 °C for about 16 h and centrifuge until a clear supernatant is obtained. The supernatant reacts with a suitable tetanus antitoxin, giving a precipitate.

into each of 5 healthy guinea-pigs, each weighing 350-450 g, that have not previously been treated with any material that will interfere with the test. The vaccine complies with the test

the test mentioned under Immunogenicity (section 2-2-3-2).

Ph. Eur. 8.3

Absence of Toxin: according to *Vaccines for vet. use 0062*, test to be performed in process immediately after detoxification but no test described

Irreversibility of Toxoid:
- Storage at 37°C over 6 weeks
- Storage at 2-8°C over 6 weeks
(control)

In vivo test requested by 0062 → free to use guinea-pigs or mice (less sensitive)

Tetanus vaccine for veterinary use (0697)

TETANUS VACCINE FOR VETERINARY USE

Vaccinum tetani ad usum veterinarium

1. DEFINITION

Tetanus vaccine for veterinary use is a preparation of the neurotoxin of *Clostridium tetani* inactivated to eliminate its toxicity while maintaining adequate immunogenic properties. The vaccine may be used to induce active and/or passive immunity.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

2-3. *MANUFACTURER'S TESTS*

2-3-1. **Absence of toxin and irreversibility of toxoid**. Carry out a test for reversion to toxicity on the detoxified harvest using 2 groups of 5 guinea-pigs, each weighing 350-450 g; if the vaccine is adsorbed, carry out the test with the shortest practical time interval before adsorption. Prepare a dilution of the detoxified harvest so that the guinea-pigs each receive 10 times the amount of toxoid (measured in Lf units) that will be present in a dose of vaccine. Divide the dilution into 2 equal parts. Keep 1 part at 5 ± 3 °C and the other at 37 °C for 6 weeks. Attribute each dilution to a separate group of guinea-pigs and inject into each guinea-pig the dilution attributed to its group. Observe the animals at least daily for 21 days. The toxoid complies with the test if no guinea-pig shows signs of disease or dies from causes attributable to the neurotoxin of *C. tetani*.

3. BATCH TESTS

3-1. **Identification**

If the nature of the adjuvant allows it, carry out test A. Otherwise carry out test B.

A. Dissolve in the vaccine sufficient *sodium citrate R* to give a 100 g/L solution. Maintain the solution at 37 °C for about 16 h and centrifuge until a clear supernatant is obtained. The supernatant reacts with a suitable tetanus antitoxin, giving a precipitate.

B. When injected into animals that do not have antibodies against the neurotoxin of *C. tetani*, the vaccine stimulates the production of such antibodies.

3-2. **Bacteria and fungi.** applicable the diluent sup with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Residual toxicity**. Administer 5 mL of the vaccine by the subcutaneous route, as 2 equal divided doses at separate sites, into each of 5 healthy guinea-pigs, each weighing 350-450 g, that have not previously been treated with any material that will interfere with the test. The vaccine complies with the test if no animal shows notable signs of disease or dies from causes attributable to the vaccine. If within 21 days of the injection any of the animals shows signs of or dies from tetanus, the vaccine does not comply with the test. If more than 1 animal dies from non-specific causes, repeat the test. If any animal dies in the 2nd test, the vaccine does not comply with the test.

3-4. **Potency**. The vaccine complies with the requirements of the test mentioned under Immunogenicity (section 2-2-3-2).

Ph. Eur. 8.3

Residual toxicity on finished product

Tetanus monographs (0697) for Vet. use: proposals for revision & alignement within Ph. Eur.

- ***absence of toxin test*** = to be performed according to 0062, in process detoxification test "*2-3-2-3. Residual live virus/bacteria and/or detoxification testing*" **but not described** then harmonised with 0452 → more sensitive test due to (next slide):
  - smaller guinea-pigs (250-350g instead of 350-450g) and
  - quantity of toxoid injected either equal or up to 14-fold higher

- ***Test for irreversibility*** of toxoid (test in guinea pigs) **deleted**
  - Rationale: Tetanus toxin was shown to lose neurotoxic activity under the conditions of the storage test at 37°C; Data confirming the stability of Tetanus toxoid (therefore no positive result ever found) →10 guinea-pigs saved per bulk purified toxoid

- ***Test for residual toxoid*** (test in guinea pigs) redundant therefore **removed**
  - Rationale: a more sensitive test for residual toxin (absence of toxin test) is performed routinely on the bulk purified toxoid + show that toxin stably detoxified → 5 guinea-pigs saved per batch

→ Alignment of Ph. Eur. monographs 0452 & 0697

Tetanus vaccine for veterinary use (0697): proposals for revision & harmonisation

Differences between both tests

As in 0452 Test more sensitive (smaller animals)

	Absence of toxin (proposed)	Residual toxicity (current)
No. of guinea pigs	5	5
Weight of guinea pigs	250-350 g	350-450 g
Injection site	subcutaneous	subcutaneous
Stage of production	*In process* test (immediately after detoxification process as requested by Monograph 0062)	Final product final bulk/final batch
Quantity injected	at least 500 Lf of the bulk purified toxoid in a volume of 1 mL	5 mL of the vaccine
Observation period for the guinea pigs	21 days	21 days
Test acceptance criteria	If within 21 days following the injection any of the animals shows signs of or dies from tetanus, the toxoid does not comply with the test. If more than 1 animal dies from non-specific causes, repeat the test once; if more than 1 animal dies in the second test, the toxoid does not comply with the test.	The vaccine complies with the test if no animal shows notable signs of disease or dies from causes attributable to the vaccine. If within 21 days of the injection any of the animals shows signs of or dies from tetanus, the vaccine does not comply with the test. If more than 1 animal dies from non-specific causes, repeat the test. If any animal dies in the 2nd test, the vaccine does not comply with the test.

the quantity of toxoid injected by administering 5 mL either equal or up to 14-fold lower than 500 Lf depending on the product.

Tetanus vaccine for veterinary use (0697) – Suppl. 10.3

European Pharmacopoeia 11.7

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EDITION 11.0: French version corrected

SUPPLEMENT 10.3

Preparation of the vaccine (section 2-1) and Residual toxicity (section 3-3). The requirement to perform the test for residual toxicity on the product as part of validation of the production process was considered redundant because a more sensitive test for residual toxin is performed routinely on the bulk purified toxoid and the revised monograph emphasises the need to validate the detoxification process to demonstrate that the toxoid is stably detoxified.
A reference to chapter *2.7.27* Flocculation value (Lf) of diphtheria and tetanus toxins and toxoids (Ramon assay) has been added.

Absence of toxin and irreversibility of toxoid (section 2-3-1). The test for irreversibility of the tetanus toxoid was no longer regarded as relevant, in view of data on the stability of the tetanus toxoid and the fact that the tetanus toxin was shown to lose neurotoxic activity under the conditions of the storage test at 37 °C. The test after storage at 5 °C, which was used as a control in the test for irreversibility, has been removed. The more sensitive test for absence of toxin carried out on non-incubated purified toxoid was retained and fully described to harmonise with the revised monograph for human use *Tetanus vaccine (adsorbed) (0452)* published in the same supplement.

SUPPLEMENT 8.3: corrected

Knowledge Database

Search Database online | Knowledge Database

Detailed view of Tetanus vaccine for veterinary use.

Status	In use
Monograph Number	00697
English Name	Tetanus vaccine for veterinary use
French Name	Vaccin tétanique pour usage vétérinaire
Latin Name	Vaccinum tetani ad usum veterinarium
Pinyin Name	
Chinese Name	
Pharmeuropa	31.2
Published in English Supplement	10.3
Published in French Supplement	11.0
On-going	Revision
State of work	2 - Pharmeuropa
Pharmeuropa	
Description	Introduction of a reference to BINACLE as an in vitro alternative to the test for Absence of tetanus toxin in guinea pigs.
Chromatogram	Not available
Additional information	Not available
History	View history
Interchangeable (ICH_Q4B)	NO
Pharmacopoeial harmonisation	NO

Reference standards

Available since	Cat. No.	Name	Batch No.	Unit Quantity	Price	SDS Product Code
	C2424400	Clostridia (multi-component) rabbit antiserum(for vaccines-vet.use) BRP	1	1 MG	90 EUR	202100087
	C2424500	Clostridium tetani guinea pig antiserum (for vaccines - vet. use) BRP	1	1 MG	90 EUR	202100088
	C2425600	Clostridium tetani rabbit antiserum (for vaccines - vet. use) BRP	1	1 MG	90 EUR	202100090

Practical Information	Test(s)	Brand Name/Information
CEP		

Tetanus vaccine for veterinary use (0697) – Suppl. 10.3

01/2021:0697

Current text

TETANUS VACCINE FOR VETERINARY USE

Vaccinum tetani ad usum veterinarium

1. DEFINITION

Tetanus vaccine for veterinary use is a preparation of the neurotoxin of *Clostridium tetani* inactivated to eliminate its toxicity while maintaining adequate immunogenic properties. The vaccine may be used to induce active and/or passive immunity.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

C. tetani used for production is grown in an appropriate liquid medium. The toxin is purified and then detoxified or it may be detoxified before purification. The detoxification process is validated to demonstrate its ability to consistently produce a toxoid that is immunogenic and stably detoxified, including at the concentration used in the final lot.

The antigenic purity is determined in Lf units of tetanus toxoid per milligram of protein and shown to be not less than the value approved for the particular product.

The toxin content (Lf per millilitre) is checked (*2.7.27*) to monitor consistency of production.

2-2. *CHOICE OF VACCINE COMPOSITION*

The vaccine is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the animals for which it is intended. The following tests for production of antigens (section 2-2-1), safety (section 2-2-2) and immunogenicity (section 2-2-3) may be used during demonstration of safety and efficacy.

The *C. tetani* strain used in the preparation of the vaccine is shown to be satisfactory with respect to the production of the neurotoxin.

2-2-1. **Production of antigens**. The production of the neurotoxin of *C. tetani* is verified by a suitable immunochemical method (*2.7.1*) carried out on the neurotoxin obtained from the vaccine strain under the conditions used for the production of the vaccine.

2-2-2. **Safety**. Carry out the test for each route and method of administration to be recommended for vaccination and where applicable, in animals of each category for which the vaccine is intended, using in each case animals not older than the minimum age to be recommended for vaccination and of the most sensitive category for the species. Use a batch of vaccine containing not less than the maximum potency that may be expected in a batch of vaccine.

For each test use not fewer than 8 animals, free from antitoxic antibodies. Administer to each animal 1 dose of vaccine. If the schedule to be recommended requires a 2nd dose, administer another dose after an interval of at least 14 days. Observe the animals at least daily until at least 14 days after the last administration.

The vaccine complies with the test if no animal shows abnormal local or systemic reactions or dies from causes attributable to the vaccine. If the test is carried out in pregnant animals, no adverse effects on gestation or the offspring are noted.

[...]

2-3. *MANUFACTURER'S TESTS*

2-3-1. **Absence of tetanus toxin**. Inject subcutaneously 1 mL containing at least 500 Lf of bulk purified toxoid into each of 5 healthy guinea-pigs, each weighing 250-350 g, that have not previously been treated with any material that will interfere with the test. If within 21 days of the injection any of the animals shows signs of or dies from tetanus, the toxoid does not comply with the test. If more than 1 animal dies from non-specific causes, repeat the test once; if more than 1 animal dies in the second test, the toxoid does not comply with the test.

2-3-2. **Batch potency test**. It is not necessary to carry out the potency test (section 3-3) for each batch of vaccine if it has been carried out using a batch of vaccine with a minimum potency. Where the test is not carried out, an alternative validated method is used, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency.

Where the test described under Potency is used as the batch potency test, the vaccine complies with the test if the antibody titre in International Units is not less than that found for a batch of vaccine shown to be satisfactory with respect to immunogenicity in the target species.

3. BATCH TESTS

3-1. **Identification**

If the nature of the adjuvant allows it, carry out test A. Otherwise carry out test B.

[...]

Monographs 0452 & 0697 in Suppl. 10.3

01/2021:0452

TETANUS VACCINE (ADSORBED)

Vaccinum tetani adsorbatum

DEFINITION

Tetanus vaccine (adsorbed) is a preparation of tetanus formol toxoid with a mineral adsorbent. The formol toxoid is prepared from the toxin produced by the growth of *Clostridium tetani*.

PRODUCTION

BULK PURIFIED TOXOID

Absence of tetanus toxin. Inject subcutaneously 1 mL containing at least 500 Lf of bulk purified toxoid into each of 5 healthy guinea-pigs , each weighing 250-350 g, that have not previously been treated with any material that will interfere with the test. If within 21 days of the injection any of the animals shows signs of or dies from tetanus, the toxoid does not comply with the test. If more than 1 animal dies from non-specific causes, repeat the test once; if more than 1 animal dies in the second test, the toxoid does not comply with the test.

01/2021:0697

TETANUS VACCINE FOR VETERINARY USE

Vaccinum tetani ad usum veterinarium

1. DEFINITION

Tetanus vaccine for veterinary use is a preparation of the neurotoxin of *Clostridium tetani* inactivated to eliminate its toxicity while maintaining adequate immunogenic properties.

and/or passive

- At least 500 Lf injected SC into 5 guinea pigs
- Animals should not show signs or die of tetanus within 21 days

2-3-1. **Absence of tetanus toxin**. Inject subcutaneously 1 mL containing at least 500 Lf of bulk purified toxoid into each of 5 healthy guinea-pigs, each weighing 250-350 g, that have not previously been treated with any material that will interfere with the test. If within 21 days of the injection any of the animals shows signs of or dies from tetanus, the toxoid does not comply with the test. If more than 1 animal dies from non-specific causes, repeat the test once; if more than 1 animal dies in the second test, the toxoid does not comply with the test.

Thank you for your attention

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EDQM Webinar 12 November 2024

BSP136

Collaborative study for the characterisation of the BINACLE assay for the *in vitro* detection of tetanus toxicity in toxoids

Part 1

Dr. Marie-Emmanuelle Behr-Gross, EDQM, Council of Europe

Presentation Outline

- BSP136 project design, timelines and revisions of tetanus vaccine monographs
- Study scope
- Study outline: design, principles and features for Part 1
- Results for Part 1
- Conclusions

BSP136 project design, timelines and revisions of tetanus vaccine monographs

Part 1

Collaborative study:	Ph. Eur. Groups 15&15V:
BSP136 endorsed in 2014	
Phase 1: completed in 2014 (37°C storage → loss of TeNT activity)	Proposal to reassess the Test for irreversibility (Sep 2015) Survey #1 with manufacturers (Nov 2015)
Phase 2: experimental phase – completed in 2016 statistical analysis – completed in 2017 procurement of material for Phase 3 1st set of pretesting results (20Lf/mL) – Dec 2018	Outcome of survey #1/Proposal to deletion (Sep 2017) Proposal to reassess the Test for specific toxicity (Mar 2018) Survey #2 with manufacturers (Jun 2018) Outcome of survey #2/revised monograph texts (Sep 2018)
Phase 2 final study report – distributed in 2019	

Part 2

Phase 3: 2nd set of pretesting results (500Lf/mL) – May 2019 procurement of material – ongoing experimental phase – in preparation	Public consultation in Pharmeuropa 31.2 (Apr 2019) Review of Pharmeuropa comments (Sep 2019) Final adoption by Ph. Eur. Commission (Nov 2019)
final prequalification of reagents and samples in 2022 collaborative study Statistical analysis Phase 3 report, etc…	

BSP136 Part 1 scope

Phase 1 (feasibility) → **Phase 2 (coll. study)**

Tests for control of:

- **irreversibility of toxoid** (0452, 0697), in-process
- **residual toxicity** (0697), batch

} omitted

- **absence of toxin** (0452, 0697), in-process → mandatory

Examine the **applicability** of the **BINACLE assay as a potential alternative to the *in vivo* test** on guinea pigs « **Absence of toxin** » **in tetanus toxoids** (0452 and 0697)

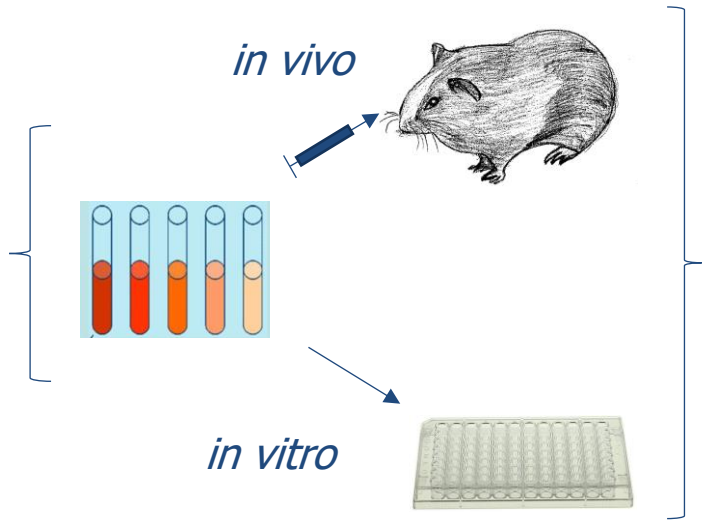
BSP136 scope cont.

How to demonstrate applicability of BINACLE as alternative to the « Absence of toxicity » compendial *in vivo* test ?

Toxoid spiked with toxin

Toxoid without toxin

Controls



Method performance

Agreement
in vivo - in vitro

BSP136 scope cont.

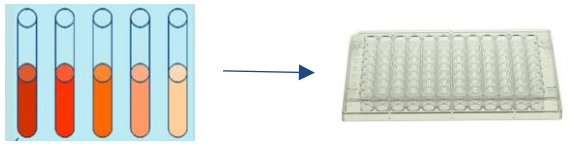
Scope of BSP136 was limited to assessing performance of the BINACLE because the *in vivo* tetanus toxicity test is a procedure qualified as « severe » for the animals

Toxoids* spiked with TeNT

Toxoids* without TeNT

Controls

*Toxoids passed *in vivo* test



Method performance
- Dose/response
- LOD
- Repeatability
- Reproducibility

5.2.14. Substitution of *in vivo* method(s) by *in vitro* method(s) for the quality control of vaccines

BSP136 Part 1 Study outline: Feasibility (Phase 1)

Select and characterise study materials

- Critical reagents (batches developed by PEI)
- Commercial reagents/materials (source batches or identify provider & reference)
- Tetanus toxoids bulks (different types/batches donated by human and vet vaccines manuf.)
- Tetanus toxin (TeNT: a centrally reserved commercial batch)

Check the performance of the BINACLE method for the purpose of the study

- Analyse and adapt the SOP developed by PEI
- Design a testing plan
- Check suitability of the assay design, readout(s) and statitical analysis model(s)

Recruit public and private sectors medicines QC laboratories involved in vaccines testing as participants to the collaborative study

BSP136 Part 1 Study outline: Collaborative study Participants (Phase 2)

Bilthovenbio	**Ceva-Phylaxia**	**USDA-APHIS-CVB**	**CZ Veterinaria**
EDQM-DLAB	**US FDA**	**GSK**	**Health Canada**
Butantan	**Merial**	**MSD Animal Health**	**NEBIH**
NIBSC	**PEI**	**Sanofi Pasteur**	**Serum institut of India**
Statens Serum Institut	**Syva**	**Zoetis**	

19 participants

QC labs (7 public sector, 12 manufacturers) from 8 European countries, Canada, India, Brazil, US

69

BSP136 Part 1 Study outline: Design of the BINACLE study (Phase 2)

Test Toxoids: 3 bulk tetanus toxoid batches (1 veterinary & 2 human products), one reference toxoid (WHO 2nd IS for tetanus toxoid for use in flocculation test; 690 Lf/ampoule)

Test conditions:

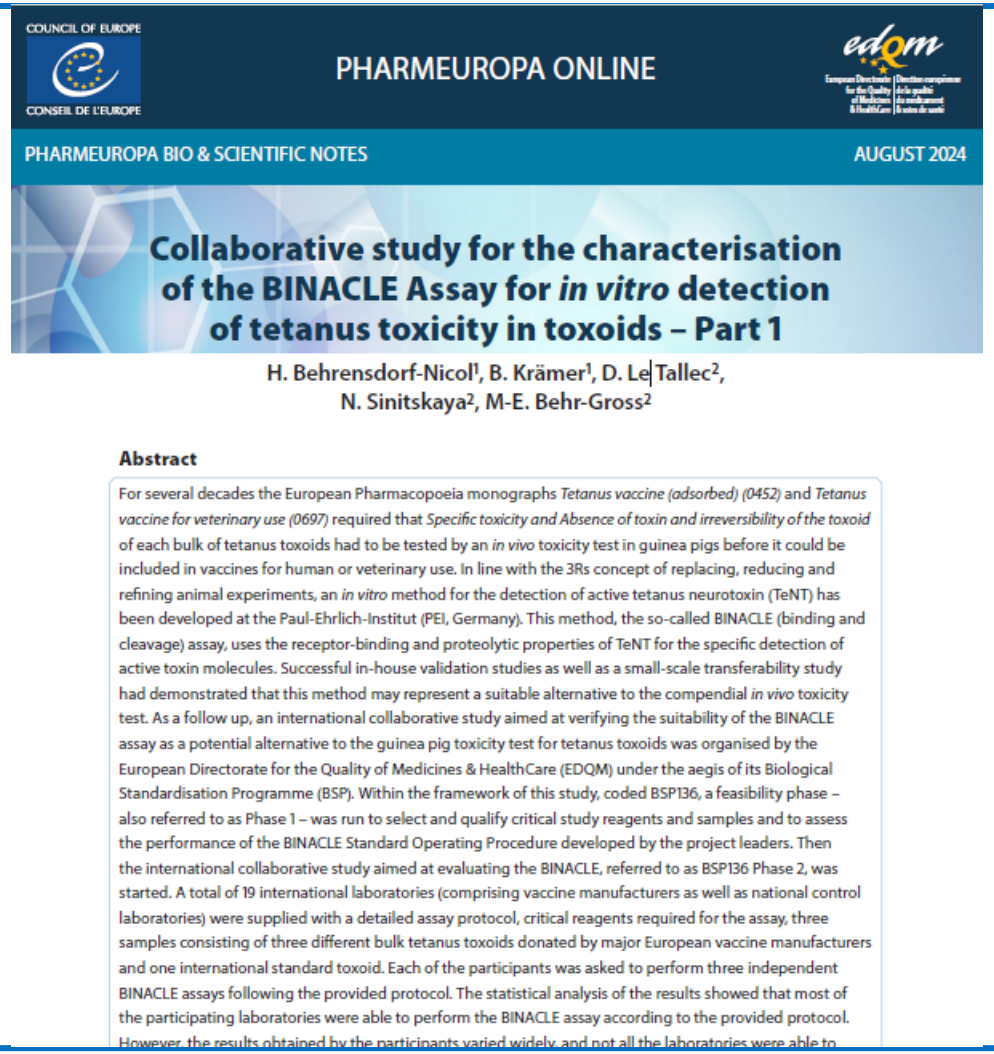
- Toxoid concentration: 20 Lf/mL
- Tetanus toxin spikes: 0.1, 0.5 and 5.0 ng/mL

Test procedure: Complex, 3 working days/assay and comprises many steps to be performed on two 96 wells plates for the binding procedure and two 96 wells plates for the cleavage procedure

Number of independent assays: 3

Test evaluation: Assessment of dose/response curves and determination of LOD for TeNT for each participant

BSP136 Part 1 Study outline: Design of the BINACLE study (Phase 2)



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

H. Behrensdorf-Nicol[1], B. Krämer[1], D. Le Tallec[2], N. Sinitskaya[2], M-E. Behr-Gross[2]

Abstract

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

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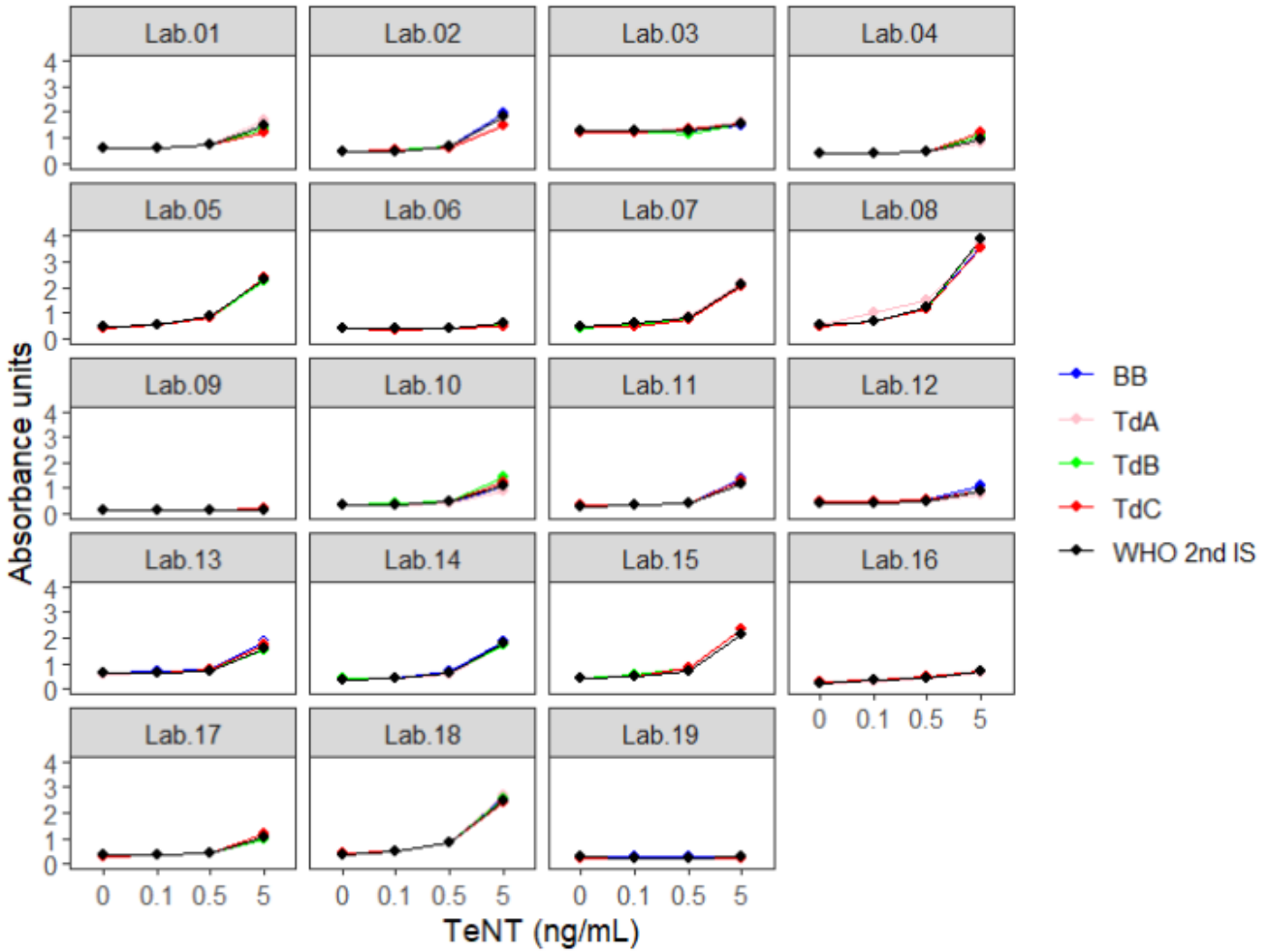
BSP136 Part 1 Study results: Number of BINACLE assays performed and testing period

Table 1 – *Overview of assays performed per laboratory*

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

BSP136 Part 1 study results: Tetanus toxin dose response curves

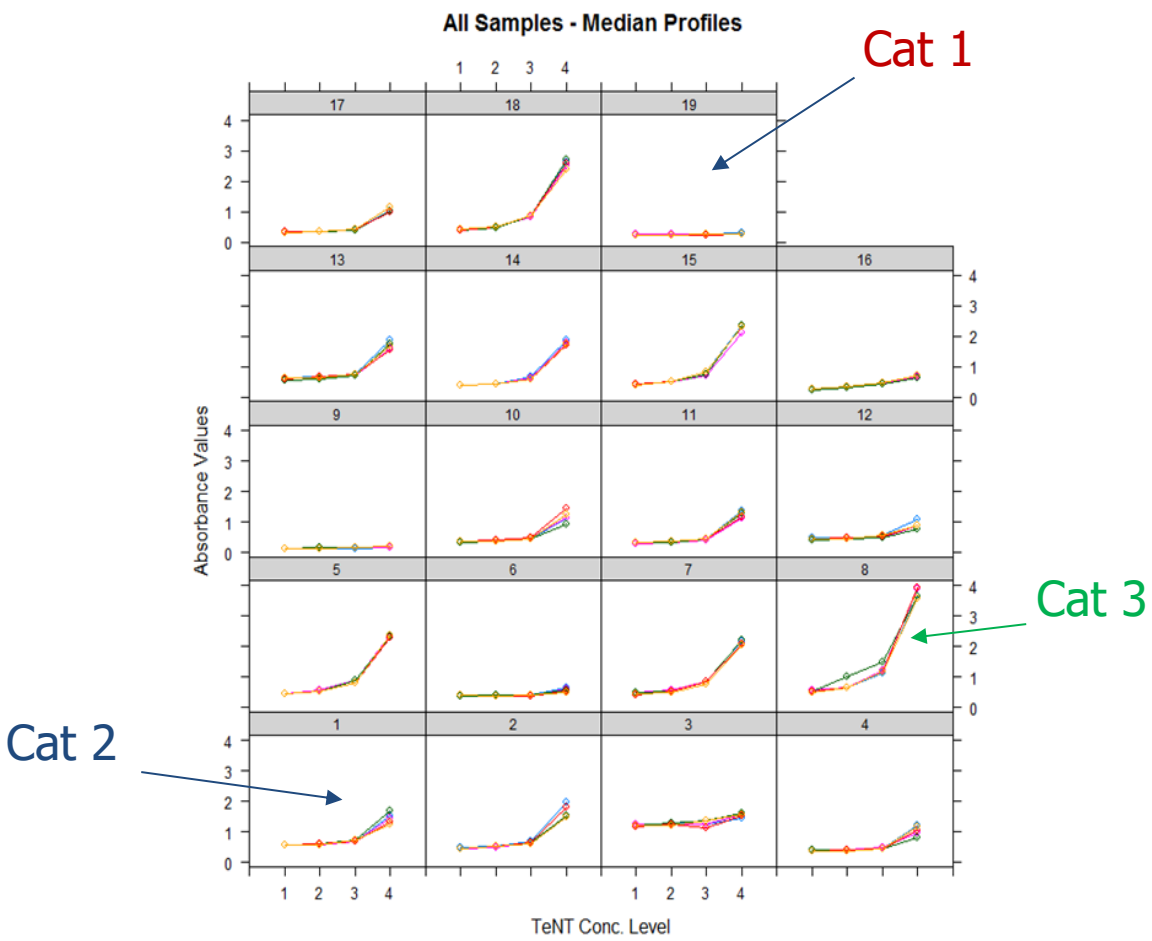
Figure 2 – *Median TeNT signal profiles obtained per laboratory and by sample*



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

The dose-response relationship for the detection of TeNT observed in BINACLE, for all toxoid assayed is identical, in all laboratories: there appears to be no matrix effect of toxoids

BSP136 Part 1 study outcome: Tetanus toxin dose response curves



According to the dose-response relationship obtained in BINACLE, **estimated by the increase in Absorbance for the concentration step 0.5-5 ng/mL** three categories of participants were distinguished:

Category 1: Low signal increase

lab. 3, 6, 9, 12, 16 and 19

Category 2: Moderate signal increase

lab. 1, 4, 5, 10, 11 and 17

Category 3: High signal increase

lab. 2, 7, 8, 13, 14, 15 and 18

BSP136 Part 1 study outcome: LOD of tetanus toxin

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

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

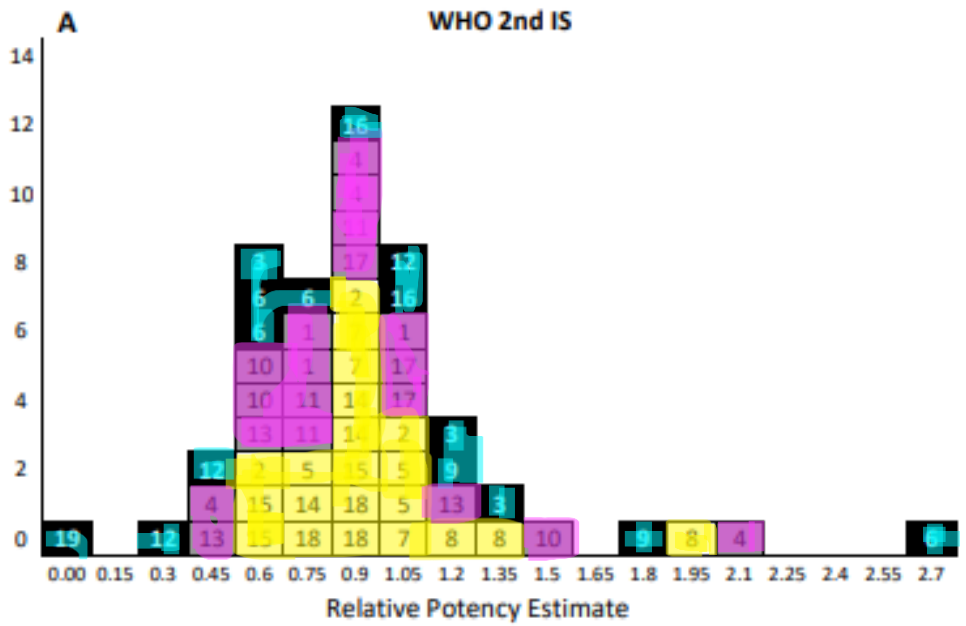
BSP136 Part 1 Study results: LOD of tetanus toxin/category

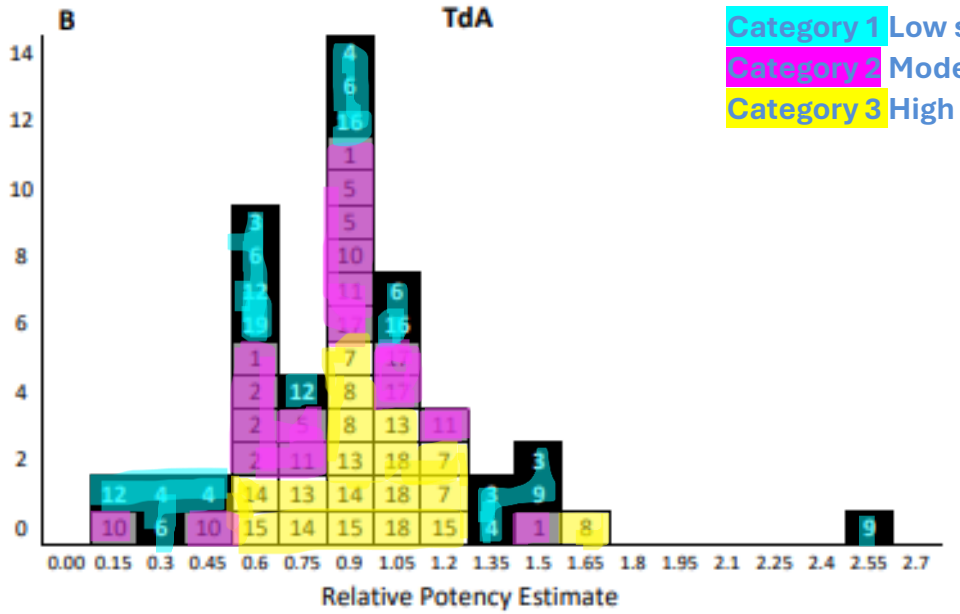
LOD expressed as TeNT concentration interval in ng/mL			
≤0.1	]0.1, 0.5]	]0.5, 5]	> 5
Lab 18	Lab 2	Lab 1	Lab 3
	Lab 5	Lab 4	Lab 9
	Lab 7	Lab 6	Lab 16
	Lab 11	Lab 8	Lab 19
	Lab 14	Lab 10	
	Lab 15	Lab 12	
		Lab 13	
		Lab 17	

Category 1 Low signal increase
Category 2 Moderate signal increase
Category 3 High signal increase

BSP136 Part 1 Study results: Relative potency estimates of tetanus toxin

A: TeNT spiked into WHO 2nd IS
B: TeNT spiked into TdA
Numbers/boxes: laboratory codes





Category 1 Low signal increase
Category 2 Moderate signal increase
Category 3 High signal increase

Histograms of potency estimates of the TeNT diluted in toxoid calculated relative to a positive control (TeNT diluted in buffer BB) for each assay performed by each laboratory

BSP136 Part 1 study outcome: Assessment of Repeatabilty and reproducibility based on RP estimates

Table 5A – *All RP estimates*

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

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

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

N Lab: number of laboratories. * N RPs: number of relative potency estimated per toxoid.
Repeatability: variability between independent assay results obtained by the same laboratory & Reproducibility: variability between independent assay results obtained by different laboratories, expressed as geometric coefficients of variation (GCV in %)

BSP136 Part 1 Conclusions

7 laboratories out of 19 generated data that shows that the BINACLE allows detecting tetanus toxin in toxoid samples in a dose-dependent manner and at a LOD which is below or comparable to that of compendial *in vivo* toxicity tests prescribed in tetanus vaccine monographs

The BINACLE has shown to be "fit for purpose" in these labs but this is less clear or not confirmed in other labs (e.g. those in category 1)

BSP136 Part 1 Conclusions

Despite that the all-over variation observed for the BINACLE in BSP136 is high when all results are considered, the repeatability and reproducibility were improved significantly when precision of measurements was taken into account (data from 4 or 5 laboratories excluded)

The fact that some laboratories failed to detect tetanus toxin in all their assays could be linked to some technical issues, to the complexity of the study and to some potentially sub-optimal characteristics of the study design and protocol

BSP136 Part 1 Conclusions

The applicability of the BINACLE assay as a potential alternative to the mandatory guinea-pigs test for the « Absence of toxin » in tetanus toxoids (0452,0697) was demonstrated for a subset of laboratories in BSP136 Part 1

Further standardisation of the BINACLE procedure and optimisation of the collaborative study design would certainly allow to provide more convincing results

=> A complementary study was undertaken

Thank you for your attention

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Challenges identified in BSP136 part 1

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Das Paul-Ehrlich-Institut ist ein Bundesinstitut im Geschäftsbereich des Bundesministeriums für Gesundheit.

The Paul-Ehrlich-Institut is an Agency of the German Federal Ministry of Health.

Paul-Ehrlich-Institut

Key conclusion from BSP136 part 1

Based on the results of **BSP136 part 1**, it was concluded that

- the **BINACLE assay** has the **potential to detect TeNT** with a **sensitivity similar to the *in vivo* test**
- with regard to the high variability of the results, an **optimised protocol** and **enhanced standardisation** would be required

Critical factors identified in BSP136 part 1

During data analysis, several **critical factors** were identified that may have contributed to the increased variability:

- Reagents
- Test samples
- Critical/complicated protocol steps
- Deviations from the protocol
- Study design

→ These factors served as **starting points for improvement measures**

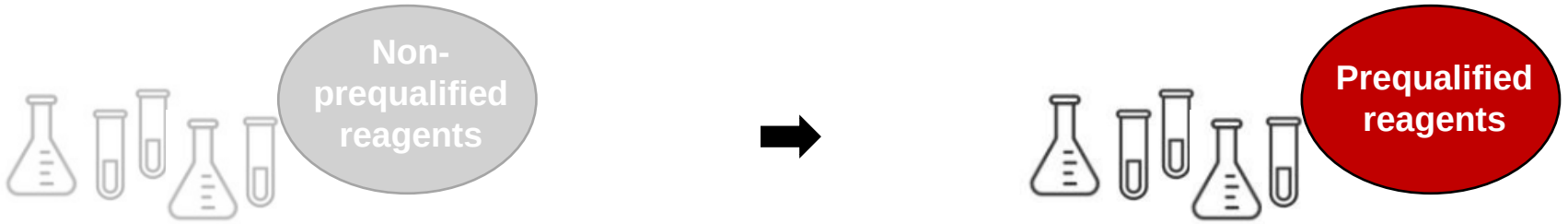
Critical factor 1: Reagents

BSP136 part 1:
Participants had to buy various reagents

- Corresponding batches were not prequalified for BINACLE suitability
- Availability of high-quality reagents may have been an issue in some cases
  (e.g. local differences: BSA used by one non-European lab was not declared as protease-free)

The use of **prequalified reagent batches** can help to ensure that the material is of **sufficient quality** and **free from impurities** that may interfere with the assay

→ **Suggested improvement:** Vast **majority of reagents should be prequalified** at the project leaders' lab and centrally provided to the study participants



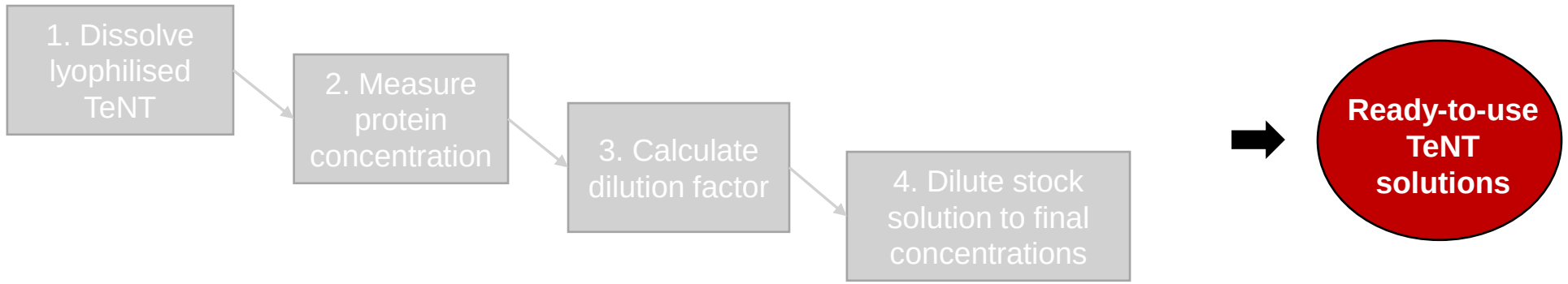
Critical factor 2: Test samples

BSP136 part 1:
Participants had to prepare the control and sample solutions

- Multi-step procedure, potentially error-prone
- All labs used the same TeNT batch as starting material, but it was not ensured that everyone ultimately had equivalent samples and controls

It is important that **all participants use equivalent samples and control solutions**

→ **Suggested improvement: Prequalified, ready-to-use TeNT solutions** should be provided to all study participants



Critical factor 3: Optimisation of protocol

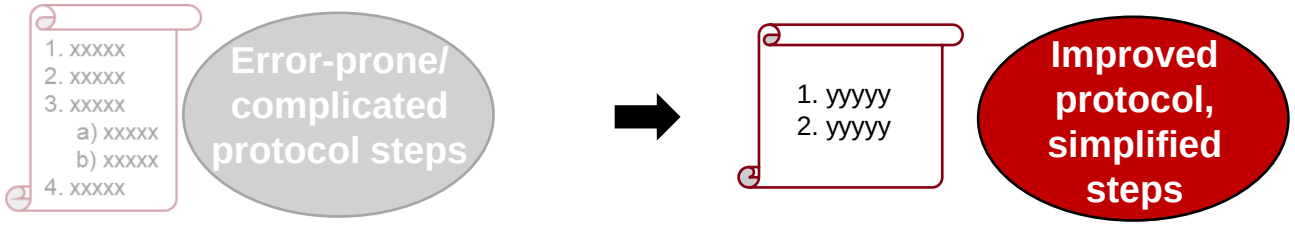
BSP136 part 1:

- While the toxin/toxoid samples were applied to the microplate according to the specified scheme, there was a risk of inappropriate drying of the remaining wells
- Several buffers and reagent solutions had to be prepared by the participants using multi-step procedures: Potential source for variability

Protocol steps that turned out to be particularly critical or complicated should be **improved and simplified** to facilitate handling and enhance standardisation

→ **Suggested measures for improvement:**

- Before sample application, some **buffer should be added** to all wells rapidly (with multi-channel pipette) to **prevent drying** of the wells
- Wherever possible, **buffers and reagent solutions** should be replaced by commercial **ready-to-use products** (e.g. PBS, TMB), or **preparation procedures** should be **simplified** (e.g. asolectin)



Critical factor 4: Deviations from the protocol

BSP136 part 1:
Several participants reported

- Deliberate **deviations from the protocol instructions** (e.g. incubation times)
- **Changes between assay runs** (e.g. reagent batches, toxin concentration)
- Accidental deviations from the protocol (e.g. pipetting errors)

To ensure **good comparability of the data**, it is important that all participants **adhere strictly to the protocol instructions**

→ **Suggested measures for improvement:**

- Participants should be provided with more **extensive information material** to minimise the risk of errors
- The importance of strict **compliance with the protocol instructions** should be **emphasized more clearly**

Critical factor 5: Study design

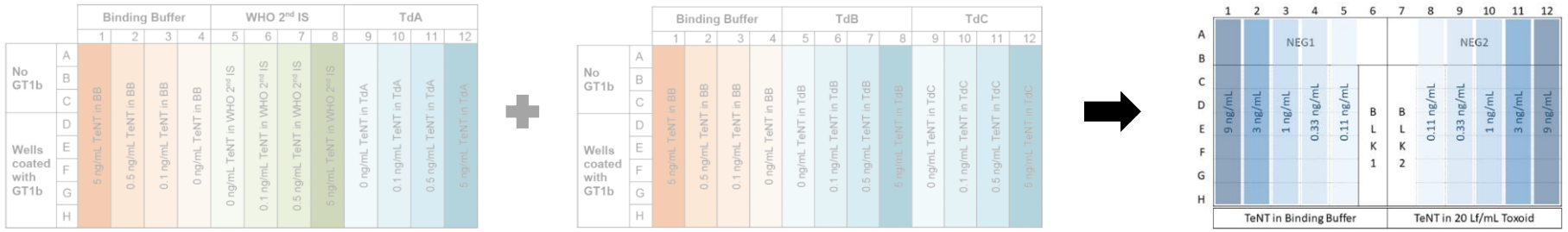
BSP136 part 1:

- Four toxoids (each in plain form + spiked with several TeNT concentrations) → many different samples, multiple microplates needed to accomodate all samples and replicates
  - Handling may have been challenging for staff with limited BINACLE experience
  - Risk of pipetting errors
- Three TeNT spike concentrations → only very rough LOD estimation

An **improved study design** could help to make the **handling less error-prone** and allow a **better estimation of the LOD**

→ **Suggested measures for improvement:**

- Only **one toxoid** should be used (so that all samples fit into one microplate)
- **Five TeNT spike concentrations** should be used



Characterisation of the improved BINACLE assay

→ The critical factors identified during BSP136 part 1 served as starting point for **optimisation of the BINACLE protocol and the study design** to reduce variability and enhance standardisation

→ The **improved BINACLE assay** was then tested in **BSP136 part 2** (also called "Phase 3") to **characterise the variability and estimate the LOD**

→ **Goal: Assess applicability** of the BINACLE assay as an alternative to the **guinea pig test for "Absence of toxin"** (Ph. Eur. monographs 0452 and 0697)

Overview: Changes for BSP136 part 2

TOPIC	CHANGES COMPARED TO BSP136 PART 1
Reagents	More prequalified reagents supplied to participants → standardisation
	Ready-to-use toxin solutions → to ensure equivalent samples and controls
	Commercial ready-to-use buffers + solutions (e.g. PBS, TMB solution) → easier handling
Protocol optimisation	Rapid addition of buffer after washing → to prevent drying of the wells
Improved plate layout	Only 1 toxoid → samples fit on one plate, easier handling
	5 instead of 3 TeNT spike concentrations → better estimation of LOD
Participants	Mainly focused on European manufacturers → to facilitate shipment of reagents
Method-related information	More information material for participants before experimental phase (Video sequences, "Recommendations" file, kick-off webinar) It was emphasized that all deviations from the protocol must be clarified in advance with the project leaders → to avoid experimental errors and deviations

THANK YOU VERY MUCH

FOR YOUR ATTENTION

BSP136

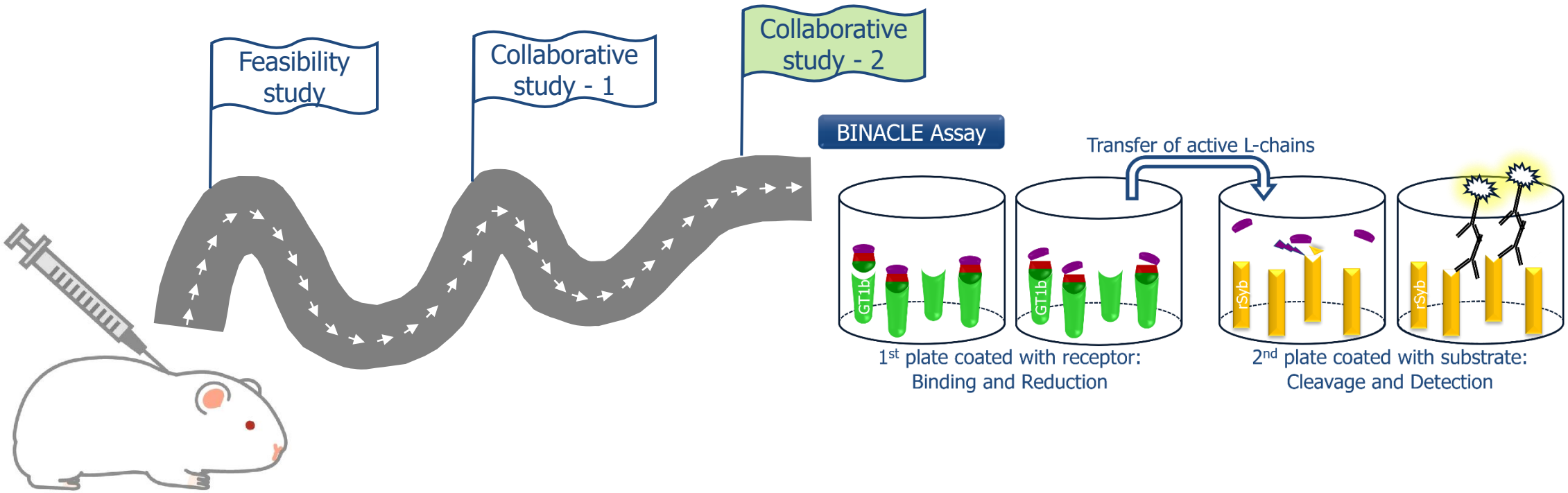
Collaborative study for the validation of the BINACLE assay for the *in vitro* detection of tetanus toxicity in toxoids – Part 2

Christina Göngrich, Biological Standardisation Programme, EDQM, Council of Europe

Contents

- Study design
- Main outcomes of the second collaborative study

  Study Aim: Estimation of LOD and characterisation of method variability



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

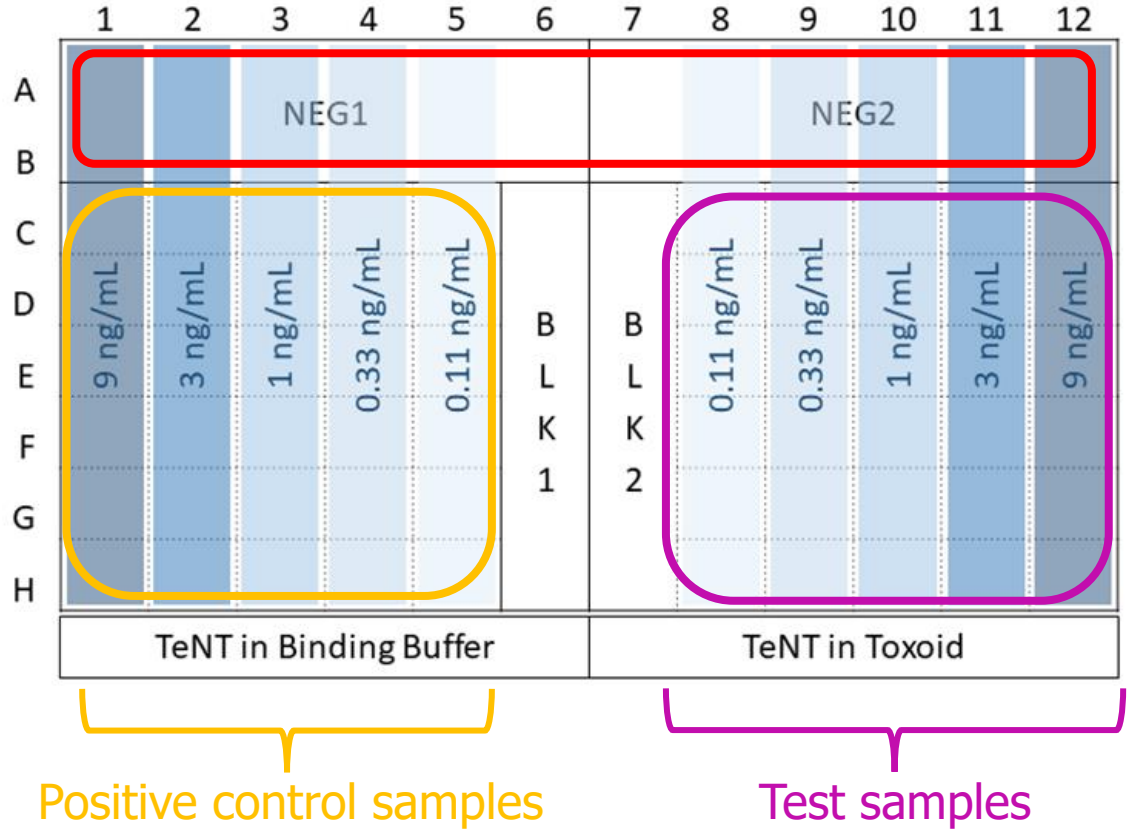
Study Design

…using an optimised and simplified study protocol and more pre-qualified reagents

- 1 toxoid, compliant with the test "Absence of tetanus toxin" Ph. Eur. *0452* & *0697*
  (20 Lf/mL = same concentration as in first collaborative study)
- 5 TeNT spike concentrations: 0.11, 0.33, 1, 3, 9 ng/mL
- 4 independent assay runs per participant
  (1 participant: 2 additional assays to check stability of Phase 3 reagents over entire experimental phase)
- 9 participants
  (4 vet vaccines manufacturers, 4 human vaccines manufacturers + PEI)
- Experimental phase carried out between February and June 2023

Samples & Controls

- Two conditions:
  - TeNT in Binding Buffer (pos. control)
  - TeNT in Tetanus Toxoid (test samples)
- Six replicate wells per TeNT concentration and condition
- Controls for unspecific binding without addition of the TeNT receptor GT1b
- Blank Controls: 0 ng/mL TeNT



Overview: Reported Data & Data Analysis

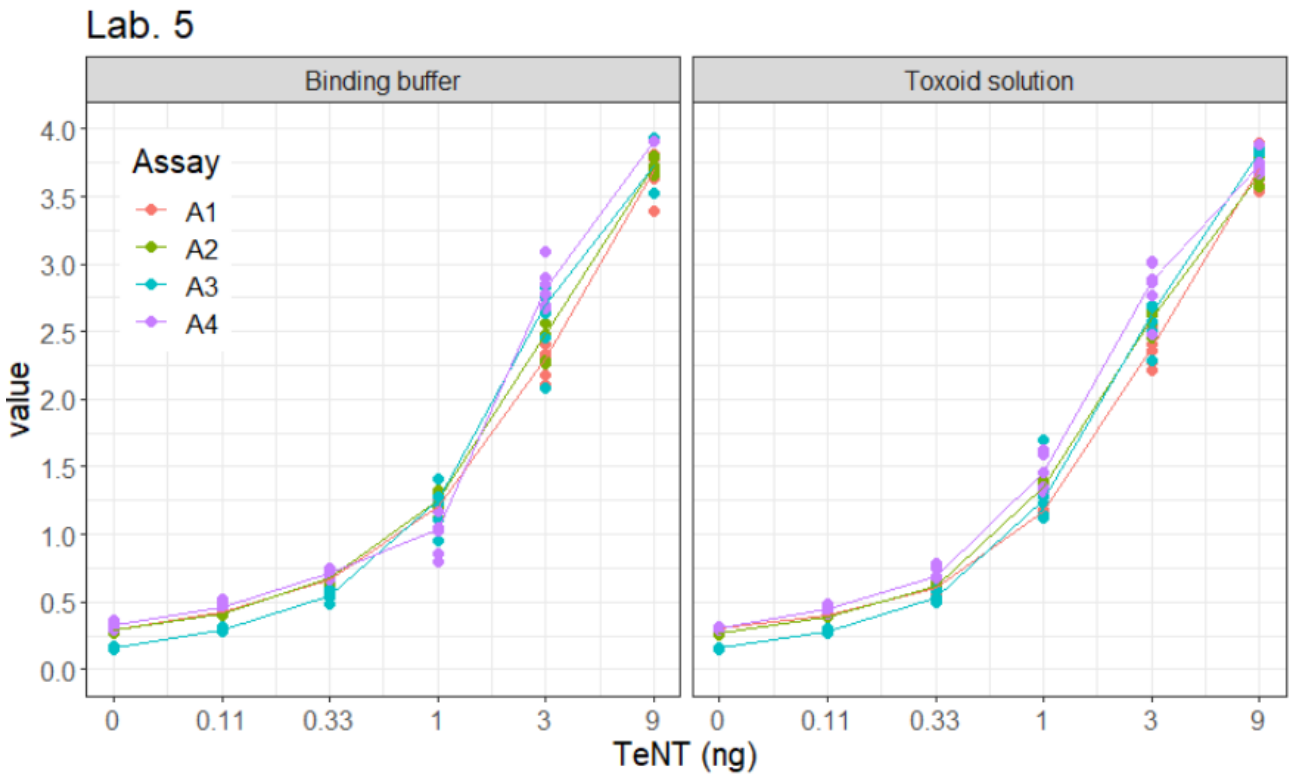
REPORTED DATA

- Absorbance values for 38 assays were reported, 2 assays were excluded due to non-fulfilment of acceptance criteria defined in the protocol → 36 assays included in analysis
- 7 individual wells were excluded due to unexpectedly high absorbance values (reference wavelength or measurement wavelength)
- Laboratory 5 reported 9 values outside of the detection range of their photometer (9 ng/mL TeNT, AU > 4.0)
  → these values were not taken into account for the statistical analysis

DATA ANALYSIS

- Estimation of method variability based on variability of relative potency estimates
- Cut-off based determination of the limit of detection (LOD)

Results (1) – Example Dose-Response Curves

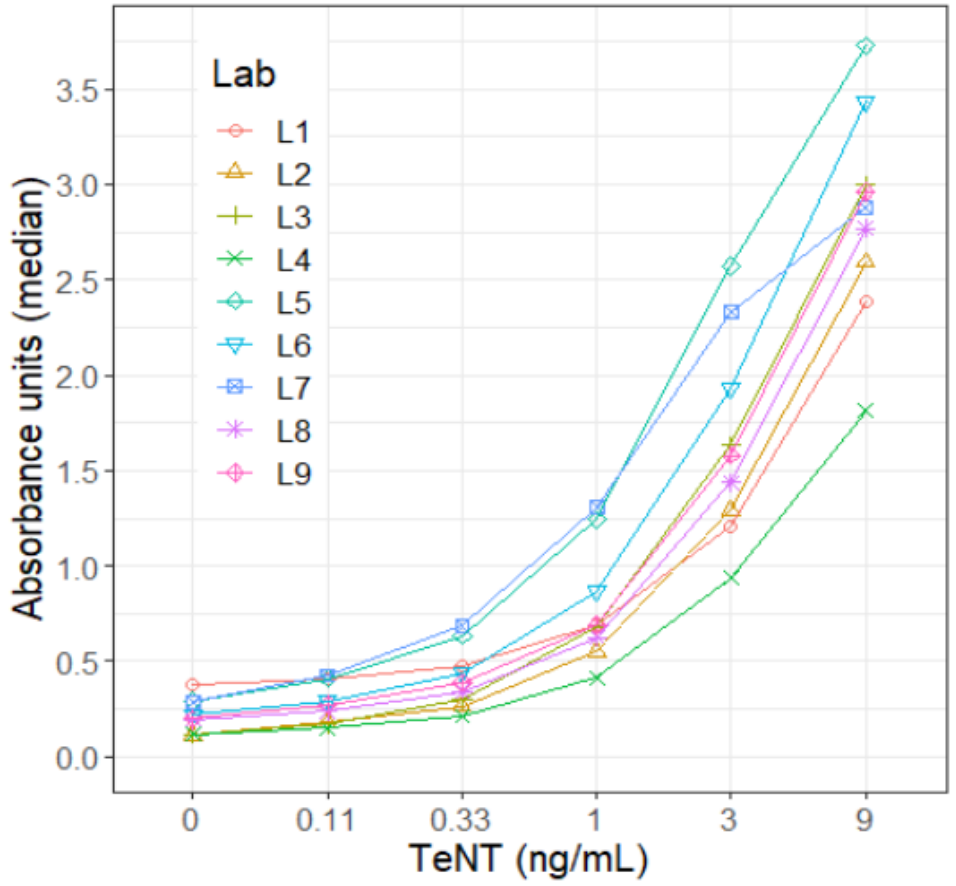


Absorbance values obtained for each TeNT concentration plotted separately for positive controls (left panel) and test samples (right panel).

- For most assays, variability between the replicate measurements for any given TeNT spike was low
- Overall, similar dose-response curves for positive control and test samples

→ The toxoid used in the study had very little impact on the detection of TeNT

Results (2) – Interlaboratory Comparison



Median dose-response curves per laboratory showing the pooled data of positive controls and test samples.

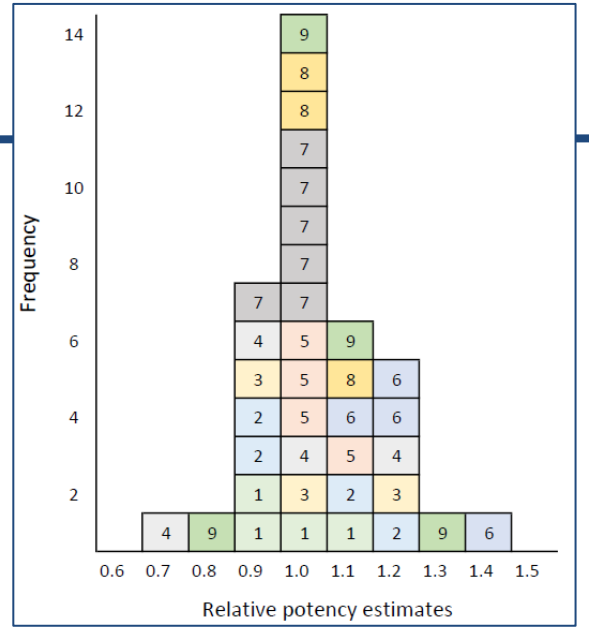
- Two laboratories obtained steep dose-response profiles (Lab 5 & 7)
- Lab 4 obtained comparatively low signal
- The remaining laboratories formed an intermediate group.

→ All laboratories could detect TeNT in a dose-dependent manner

Results (3) – Method Variability

$RP = ED_{50(\text{test samples})} / ED_{50(\text{positive control samples})}$

Variability of the RP values calculated by ANOVA



- Intra-laboratory GCV (average): 12 % GCV

Laboratory	5	7	8	1	6	3	2	9	4
GCV	3 %	5 %	5 %	9 %	10 %	12 %	16 %	19 %	20 %

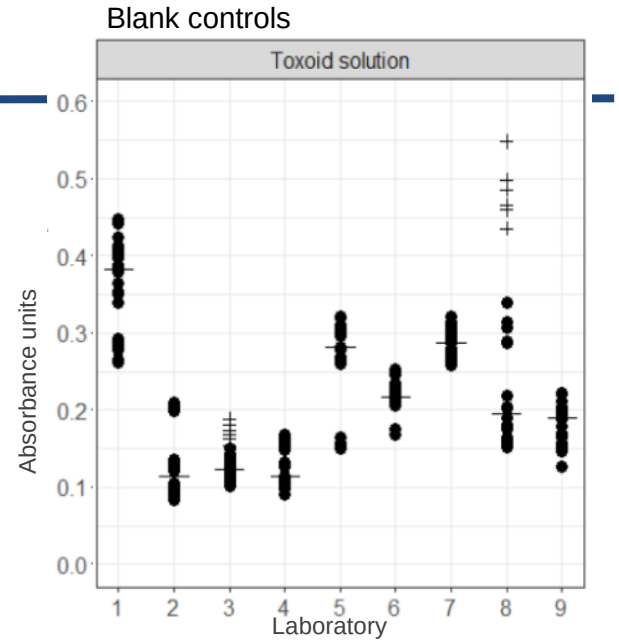
- Inter-laboratory GCV: 4 %
- Reproducibility (intra + interlaboratory variability): 13 % GCV

→ Variability was similar to commonly reported values for immunochemical assays

Results (4) – Limit of Detection

$$\text{Cut-off} = \text{Mean}_{BLK} + 3.3 \times \text{SD}_{BLK}$$

$$\text{LOD}_{ASSAY} = [\text{TeNT}] \text{ for which } > 80\% \text{ of values are above cut-off}$$



LOD_{ASSAY}

	TeNT in Binding Buffer [ng/mL]					TeNT in Toxoid [ng/mL]				
	9	**3**	**1**	**0.33**	**0.11**	**9**	**3**	**1**	**0.33**	**0.11**
Assays Above Cut-off [%]	100	100	100	97	61	100	100	100	97	69

- 0.33 ng/mL TeNT detected in 35 out of 36 valid assays
- 0.11 ng/mL TeNT detected in > 60 % of all valid assays
- LOD was 0.11 ng/mL for at least 1 assay in all laboratories

Results (5) – Limit of Detection

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

LOD_{ASSAY} = [TeNT] for which > 80% of measurements above Cut-off

LOD_{LAB} = [TeNT] for which 3 of 4 assays above Cut-off

LOD_{LAB}

Laboratory	1	2	3	4	5	6	7	8	9
TeNT in Toxoid [ng/mL]	0.33	0.11	0.33	0.11	0.11	0.11	0.11	0.33	0.33

✓ 5 Laboratories: LOD 0.11 ng/mL TeNT

✓ 4 Laboratories: LOD 0.33 ng/mL TeNT

→ Close agreement of the results between laboratories

→ *In vitro* LOD in the range of the expected *in vivo* detection limit

Conclusions

- For BSP136-Part 2, the study design and protocol were optimised with the aim to reduce variability and to better define the LOD.
- The results of BSP136-Part 2 demonstrated acceptable variability of the BINACLE method.
- The detection limit of the BINACLE method for the tested TeNT batch and toxoid was in the range of the estimated detection limit of the animal test.
- With appropriate in-house validation by the user, the BINACLE method has the potential to replace guinea pigs in the safety testing of tetanus vaccines.

→ Proposal of the BINACLE to Ph. Eur. expert groups 15 and 15V for consideration as an alternative method to the current *in vivo* test.

Project Contributors

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&
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Collaborative study participants & the laboratory staff actively involved in performing the tests

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COUNCIL OF EUROPE
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The Paul-Ehrlich-Institut for donation of the cleavage site specific antibody & recombinant synaptobrevin

Thank you for your attention

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Considerations for method validation and implementation

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Agenda

- Toxoid Concentration
- Limit of Detection
- Reagents
- Suggestions for Validation and Routine Use

TOXOID CONCENTRATION

Toxoid concentration used in BSP136

In **BSP136, toxoid solutions** were tested in **20 Lf/mL** concentration

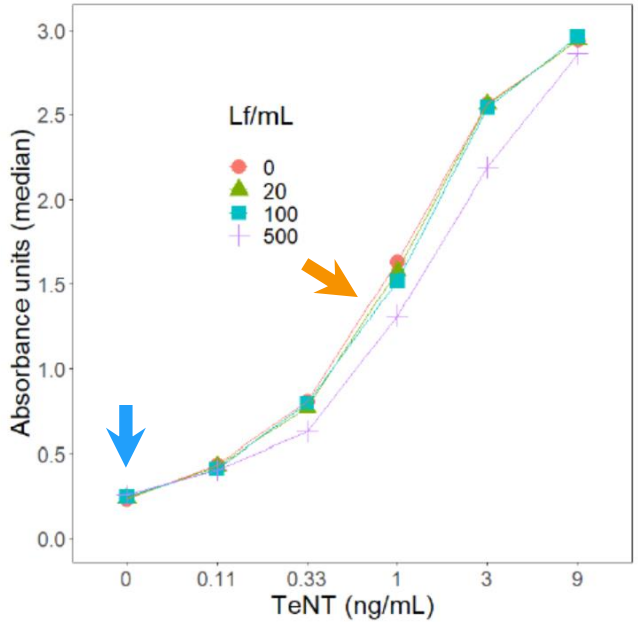
- **Part 1:** 20 Lf/mL chosen based on relevance for test for "irreversibility of toxoid"
- **Part 2:** 20 Lf/mL chosen for good comparability of results between both phases

  (Main aim of part 2: Demonstrate improved performance of optimised BINACLE compared to original protocol)

- In the meantime: **Irreversibility test was deleted** from Ph. Eur.
- Presently prescribed **animal test for "absence of toxin"** (Ph. Eur. 0452 and 0697) uses **concentrated toxoid solutions** (≥ 500 Lf/ml)

→ **Applicability of optimised BINACLE assay to concentrated toxoids** was examined at PEI in parallel to BSP136 part 2

BINACLE testing of concentrated toxoids



[Figure from: Behrensdorf-Nicol H, Le Tallec D, Sinitskaya N, Behr-Gross ME, Göngrich C (2024) Pharmeur Bio Sci Notes 2024:162-192]

Toxoid	LOD [ng/mL TeNT]
0 Lf/mL	0.11 in 3 out of 3 assays
20 Lf/mL	0.11 in 3 out of 3 assays
100 Lf/mL	0.11 in 3 out of 3 assays
500 Lf/mL	0.11 in 2 out of 3 assays

BINACLE results (same toxoid and TeNT batches as in BSP136 Part 2):

Increasing toxoid concentrations had only limited impact on

- background signal (blank control)
- dose-response curves for TeNT
- the LOD

→ For toxoids that are well suitable for BINACLE testing, it is expected that the test yields **comparable results when carried out using 20 Lf/mL or 500 Lf/mL toxoid**

LOD_{ASSAY} = [TeNT] for which > 80% of values are above cut-off

Cut-off = $Mean_{BLK}$ + 3.3 x SD_{BLK}

LIMIT OF DETECTION

Recommendation from European Pharmacopoeia

Ph. Eur. monograph 5.2.14 "Substitution of in-vivo method(s) by in-vitro method(s) for the quality control of vaccines":

"An *in vitro* method for detection of residual toxic components should be specific and at least as sensitive as the existing *in vivo* method. …

In most cases, where an *in vivo* assay is to be replaced there will be data available on the sensitivity of that model for detection of the toxin in question. Therefore, new *in vitro* methods can be characterised to demonstrate that they are sufficiently sensitive using spiking experiments and referring to historic data for the *in vivo* assay."

Estimated LOD of *in vivo* assay

Literature data about LOD of the guinea pig assay is scant and variable

- MLD in guinea pigs: 0.3 ng/kg*
  - Very rough estimation based on data from several older reports (TeNT preparations of different purity, different routes of administration)
  - Extrapolated from data obtained in mice, questionable conversion factor
- Severe symptoms in guinea pigs: 1.5 ng/kg#
  - Based on 2 guinea pig tests performed by a vaccine manufacturer
  - TeNT diluted in 20 Lf/mL toxoid
  - Same administration route as for Ph. Eur. test (s.c. injection)

→ Difference probably attributable to:

- Animal species (older report: based on mouse data)
- Routes of administration
- Purity / specific activity of TeNT preparations
- Presence or absence of toxoid

* Gill DM, Bacterial Toxins: A Table of Lethal Amounts, Microbiological Reviews, 1982
# Krämer B et al., Entwicklung einer in vitro Methode zur Bestimmung von Tetanus-Toxizität: Schlussbericht, Technische Informationsbibliothek, 2007

Comparison of *in vivo* and *in vitro* LOD

Approximate LOD comparison (rough estimation, as *in vivo* LOD is not exactly known)	
Ph. Eur. *in vivo* test	**BINACLE assay**
Estimated LOD (historic literature data): 0.3-1.5 ng/kg Ph. Eur. specifications: Guinea pig weight 250-350 g, injection volume 1mL → Expected to **detect toxoid solutions containing at least 0.1 to 0.5 ng/mL TeNT**	→ Shown in BSP136: **Detects toxoid solutions containing 0.1 to 0.3 ng/mL TeNT**

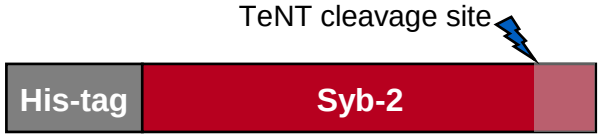
→ Based on the BSP136 results, we assume that the **LOD of the BINACLE assay** is **approximately equivalent to the estimated LOD of the guinea pig test**

→ Therefore, we are confident that **toxoid bulks failing the *in vivo* test** can also be **reliably detected in the BINACLE assay** (provided that the toxoids can be used at the same concentration in the *in vitro* and *in vivo* tests)

REAGENTS

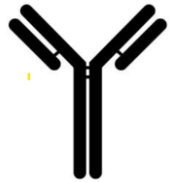
Critical reagents

Recombinant synaptobrevin-2



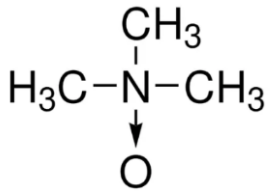
- Produced by PEI or by a CMO (expression plasmid donated to PEI)
- Important: Correct folding and length (at least amino acids 2 – 94)
- Protocol for production is published [Pharmeuropa Bio & Scientific Notes (2024) 162-192]
- Some commercially available products are also suitable

Antibody specific to cleaved synaptobrevin-2



- Rabbit polyclonal antibody
- Manufactured by a CMO, immunisation protocol developed by PEI
- Protocol for production is published [Toxicology in Vitro 21 (2007) 1641-1649]

Trimethylamine-N-oxide (TMAO)



- Osmolyte stabilising proteins, added to synaptobrevin cleavage step to increase toxin signals
- Commercially available, but: many commercial batches are of insufficient quality

Reference material

Reference TeNT

- No internationally accepted reference TeNT available
- In-house reference TeNT can be used instead
  - In-house reference preparation should be established in parallel to method validation
  - New batches can then be qualified in the BINACLE assay to ensure consistency with the previous batch

In-house reference toxoid

- To be established during manufacturer-specific method validation
- Well-characterised toxoid bulk previously shown to comply with Ph. Eur. specifications
- Representative of the vaccine antigen ("typical" bulk, manufactured at the same site and using the same protocol as for the test toxoids)
- Does not interfere with sensitive toxin detection, therefore allows reliable detection of insufficiently detoxified toxoids

SUGGESTIONS FOR VALIDATION AND ROUTINE USE

Validation of the BINACLE assay

Before the BINACLE assay can be used for batch testing purposes, **users need to validate the method for their specific toxoid product**

Key point:

It must be shown that **non-compliant toxoid bulks** are **reliably detected**

→ To ensure that the **switch to *in vitro* testing** has no negative impact, but rather **contributes to high product quality + safety**

Suggestions for validation

Step 1: Characterise **suitability of the specific toxoid product** for BINACLE testing

- Elaborate dose-response curves for several toxoid bulks compliant with the monographs and check if they induce elevated **background levels** compared to the buffer control
- Define **suitable toxoid concentration** for BINACLE testing
  - If concentrated toxoids do not cause elevated background, 500 Lf/mL could be chosen (analogous to *in vivo* test)
  - Otherwise, determine highest toxoid concentration that elicits signals similar to the buffer control (slightly elevated background level may be acceptable if it does not interfere with sensitive TeNT detection)
- Assess **batch to batch variability** of the toxoid signals

Suggestions for validation (cont.)

Step 2: Determine ***in vitro*** **LOD** and **compare to *in vivo* test**

- Spike toxoids characterised in step 1 with reference toxin to **determine the LOD of the BINACLE assay** for active TeNT
- **Compare to LOD of *in vivo* test** for the same toxin preparation (e.g. using historical in-house data)
  Any **differences between the tests** (e.g. toxoid concentration, sample volume) must be **taken into account** for this comparison!
- Check that both tests yield the same result regarding **rejection or acceptance** of a sample

Suggestions for routine use: Plate layout

For **routine BINACLE testing after validation** of the method, the plate layout can be adapted to the individual needs

We suggest that the following recommendations should be observed:

- Each sample and control solution should be applied to the plate in **several replicates**
- In addition to the **test toxoid(s)**, **controls** should be included on each microplate to check if the assay worked well and shows an appropriate LOD for active toxin:
  - **Buffer** (without toxin or toxoid)
  - In-house **reference toxoid** (without TeNT)
  - Several dilutions of **reference TeNT** (concentration range should **cover the LOD of the method** as established during validation)
    - **in reference toxoid**
    - **in buffer**
  - In some cases, additional controls (e.g. without receptor or without anti-synaptobrevin antibody) might be useful

Example plate layout for routine testing

Example plate layout for routine testing (non-binding proposal):

	1	2	3	4	5	6	7	8	9	10	11	12
A	Buffer + TeNT* conc. 1 (high conc.)				Ref. Txd§ + TeNT* conc. 1 (high conc.)				Space for additional controls			
B	Buffer + TeNT* conc. 2				Ref. Txd§ + TeNT* conc. 2							
C	Buffer + TeNT* conc. 3				Ref. Txd§ + TeNT* conc. 3				Test Toxoid A#			
D	Buffer + TeNT* conc. 4				Ref. Txd§ + TeNT* conc. 4				Test Toxoid B#			
E	Buffer + TeNT* conc. 5				Ref. Txd§ + TeNT* conc. 5				Test Toxoid C#			
F	Buffer + TeNT* conc. 6 (low conc.)				Ref. Txd§ + TeNT* conc. 6 (low conc.)				Test Toxoid D#			
G	Buffer without TeNT				Ref. Txd§ without TeNT							
H	Space for additional controls											

* Reference TeNT, § In-house reference toxoid

Test Toxoids: applied in same concentration as the reference toxoid (e.g. 500 Lf/mL, if possible)

Suggestions for routine use: Validity and acceptance criteria

Validity criterion:

- A BINACLE test can be considered valid if the **LOD for the reference TeNT** is **consistent with the LOD established during validation**

Acceptance criterion:

- A tested toxoid bulk complies with the test if the **signal measured for the toxoid does not exceed the cut-off value** determined for the in-house reference toxoid without added TeNT in the same assay

THANK YOU VERY MUCH

FOR YOUR ATTENTION