BINACLE Assay for Tetanus Neurotoxin: Outcomes of Project BSP136

Session II: Towards implementation of the BINACLE for QC testing of tetanus vaccines

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EDQM, Council of Europe

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EDQM webinar

Towards the introduction of BINACLE in Ph. Eur. tetanus vaccine monographs

12 November 2024

Gwenaël Ciréfice & Catherine Lebrun, EDQM

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0452 & 0697 in Supplement 10.3 (January 2021)

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 conta… of 5 h… have … interf… of the… does … from … 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. The vaccine may be used to induce active and/or passive immunity.

2-3. *MANUFACTURER'S TESTS*

2-3-1. **Absence of tetanus toxin**. Inject subcutaneously … into … , that … t will … n any … toxoid … lies … than 1 animal dies in the second test, the toxoid does not comply with the test.

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.

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Towards the introduction of BINACLE in 0452 & 0697

Background considerations

- Tetanus neurotoxin (TeNT) is extremely potent (lethal dose for human and many animals around 1-10 ng/kg body weight)
- Need for a reliable safety test for TeNT detection
- The current Ph. Eur. test using guinea pigs is the official method

- The sensitivity of the *in vivo* test is not fully established (0.1–1 ng/mL TeNT?)

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Towards the introduction of BINACLE in 0452 & 0697

Background considerations

- BINACLE may not be suitable for all toxoids (*cases where there is a high background signal*) but it is to date the only alternative available
- Part 2 of the BSP study was successful with 9 participating laboratories, using pre-qualified reagents and optimised protocol

→ **1)** clear dose-response curve ☑
2) 0.11 ng/mL TeNT detectable ☑
3) Variability (intra & inter-lab) 13% ☑

→ **What consequences for Ph. Eur. monographs?**

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Towards the introduction of BINACLE in 0452 & 0697

Additional considerations

- **Applicability of BINACLE**
  - With the current protocol, certain toxoids may not be amenable to BINACLE testing due to high background signal
    - → Alternative method, cannot fully replace the test in guinea pigs
    - → Product-specific validation is required
- **Validation of BINACLE**
  - Introduction of BINACLE requires product-specific validation:
    - establishment of the suitability of the method for the specific toxoid
    - comparison of sensitivity with that of the test in guinea pigs
- **Reagents**
  - Procurement of critical reagents Synaptobrevin and anti-Synaptobrevin antibody that are not commercially available

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Next step: Pharmeuropa teaser

Ph. Eur. experts groups 15 & 15V* discussed this jointly

No full replacement

- **Need for a product-specific validation** to demonstrate that toxoids from the routine production process do not interfere with sensitive detection of tetanus neurotoxin
- No background signal

☺ Open to alternative methods

☹ *In vivo* test still in the Ph. Eur.

Discussions

- Should it be open to **any alternative *in vitro* method** to the guinea pig test to show absence of tetanus toxin?
- What **level of detail should be included** in the Ph. Eur. for BINACLE? (E.g. validity & acceptance criteria?)
- Should the alternative method **mimic** the specific steps of the mechanism of action of tetanus toxin?
- **Product-specific validation** to be described (e.g LOD and comparison of *in vivo* and *in vitro* sensitivity)?
- Need to consider the impact on the *in vitro* test at time of significant change to the manufacturing process ?

☺ 3Rs → any validated alternative method included?

☺ BINACLE based on a published collaborative study the only to date

☹ Only 1 alternative method available at the moment

3Rs

- According to Directive 2010/63/EU, BINACLE or other alternative methods **must** be used if shown suitable

☺ Save animals with at least the same level of safety for the products

☹ Except for products with high background in BINACLE

*

15	Human Vaccines and Sera
15V	Veterinary vaccines and sera

Outcome of the discussions in Pharmeuropa 37.2 – stay tuned!

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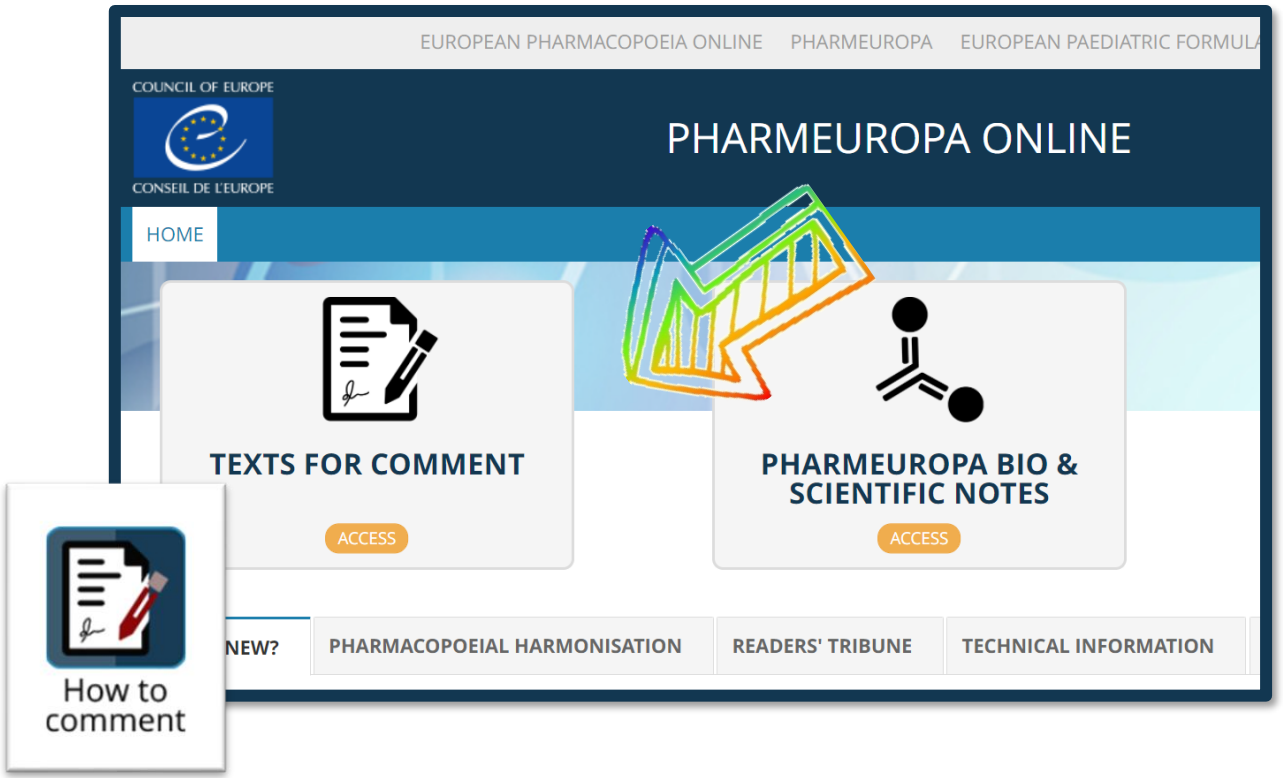
Pharmeuropa

Pharmeuropa 37.2
Commenting period from April 2025:
- to 30 June 2025 for the public
- to 31 August 2025 for the NPA



Your opinion counts, do not forget to take part to the public enquiry

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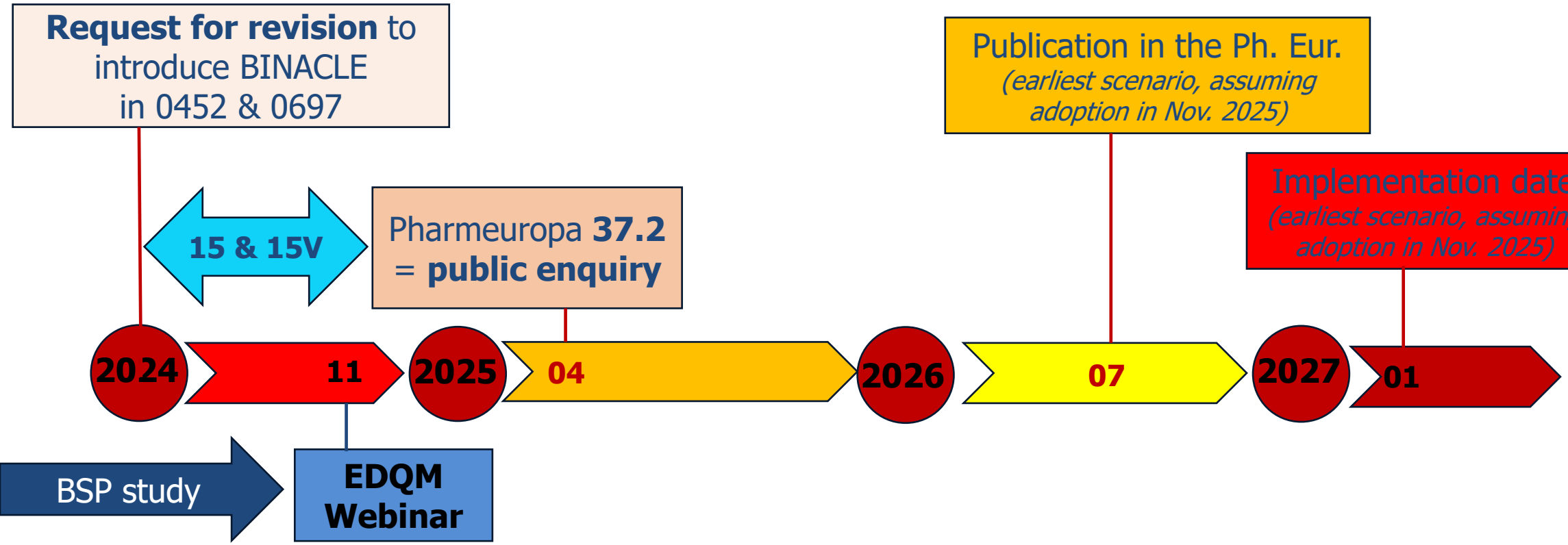
https://pharmeuropa.edqm.eu/home

Envisaged timelines for revision of Tetanus vaccine monographs



Revision of Ph. Eur. monographs 0452 & 0697 on *Tetanus vaccines for human and veterinary use*

to introduce a reference to the BINACLE assay (Binding **A**nd **Cle**avage assay**) as an alternative *in vitro* method to the test for Absence of tetanus toxin in guinea pigs carried out on each bulk purified Tetanus toxoid**

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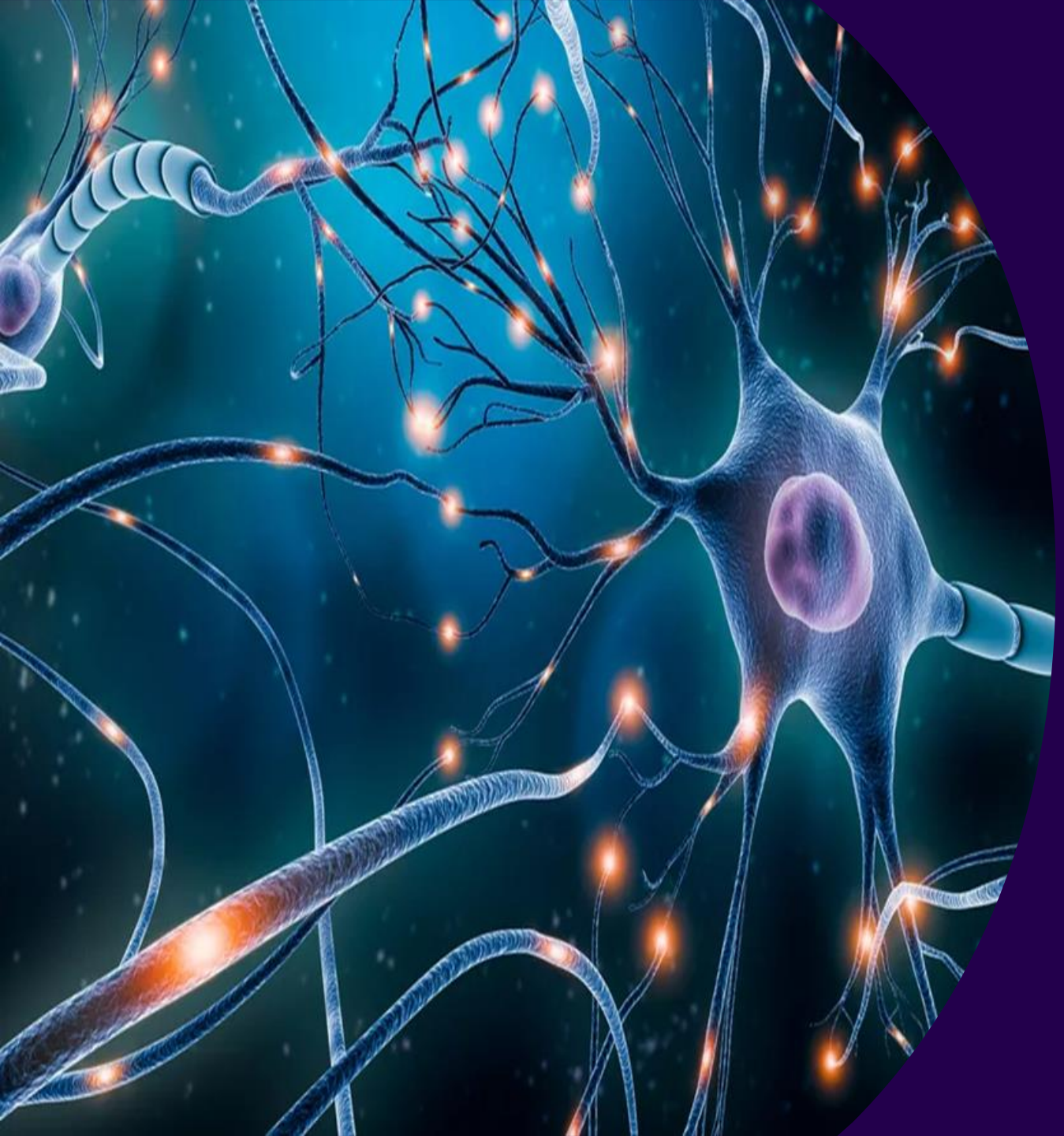


sanofi

BINACLE Assay for Tetanus Neurotoxin: Optimization Perspectives for QC use

EDQM Webinar

Romain PIZZATO, Principal Scientist
Immunology, Analytical Sciences, Sanofi
France

November 12, 2024

AGENDA

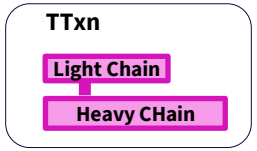
1 Optimization of the BinACle assay

2 Data obtained with optimized assay

3 Remaining challenges for next steps

BinACLe : principle and optimization

TTxn: Tetanus Toxin
TTxd: Tetanus Toxoid



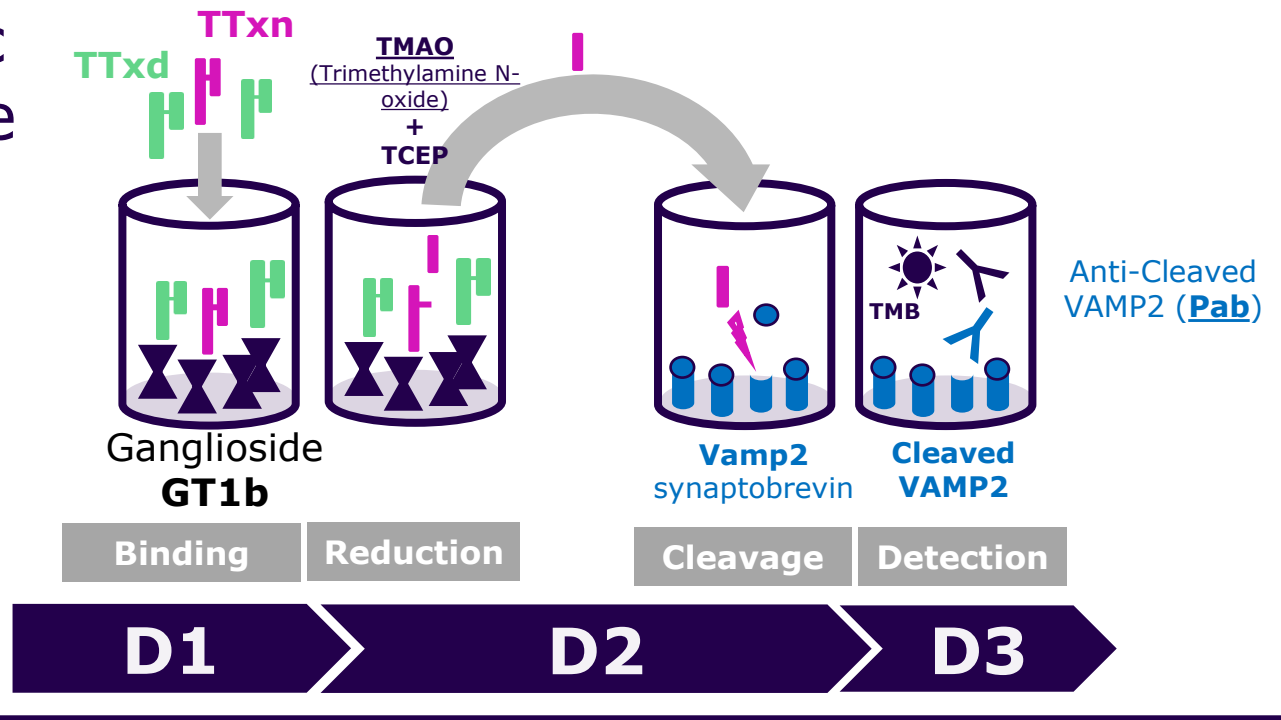
Optimized Binacle

Enz. Reaction : TMAO ⇒ Betaine (reliable non CMR alternative)

GT1b: Animal extract ⇩ synthetic

On going

Classic Binacle



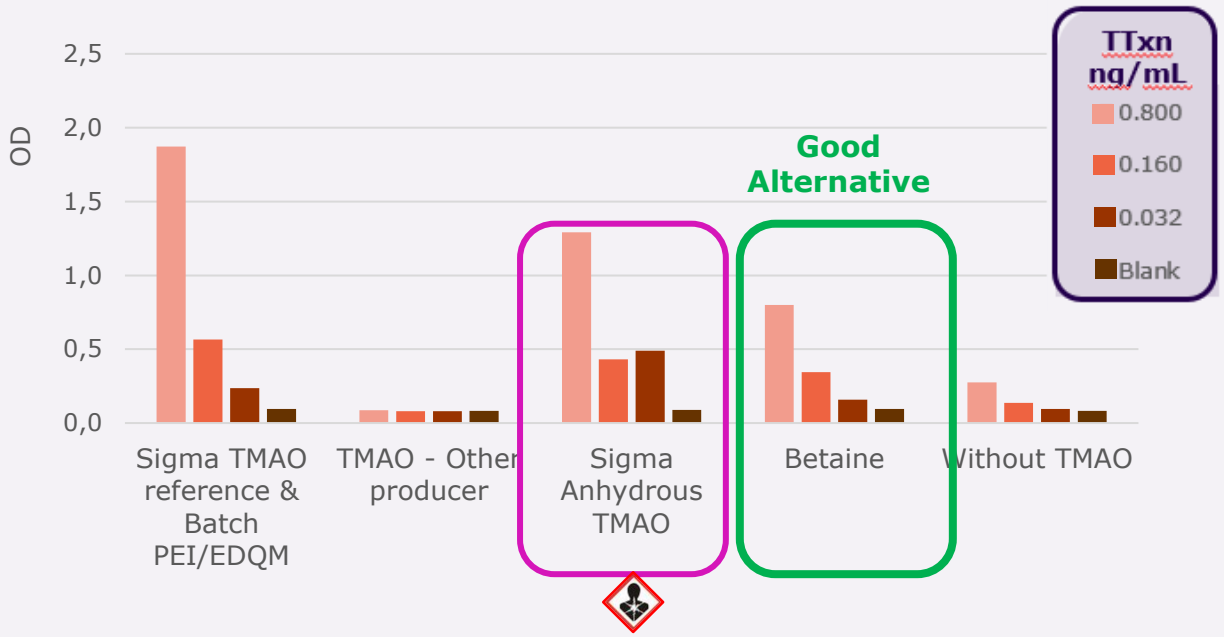
Pab ⇒ Mab

D1 D2

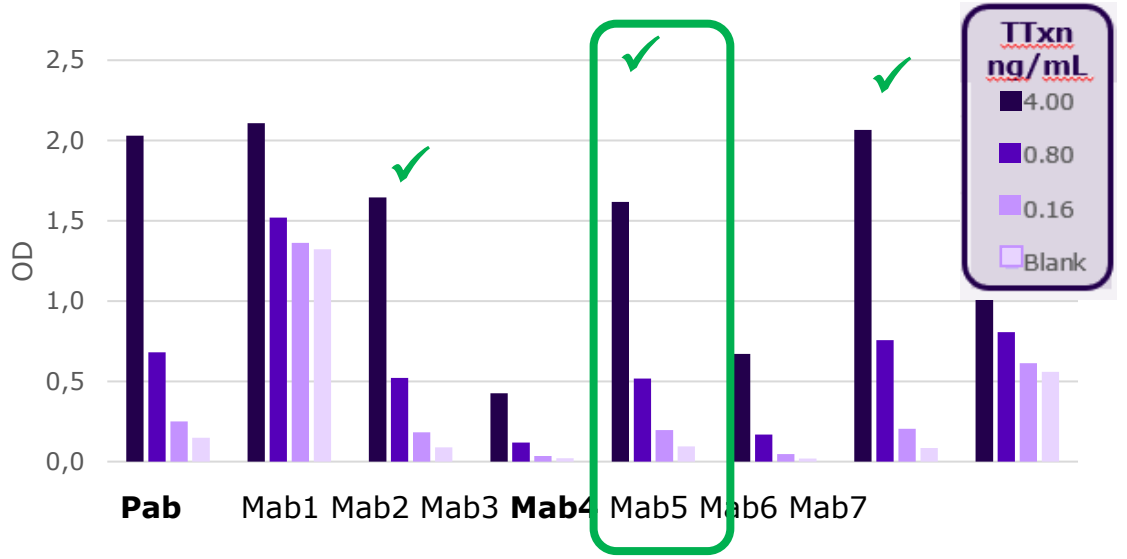
BinACLe : optimization Data

Toxin detection (signal) measured with alternative critical reagents (TMAO & Mab)

TMAO Alternatives



Pab replacement

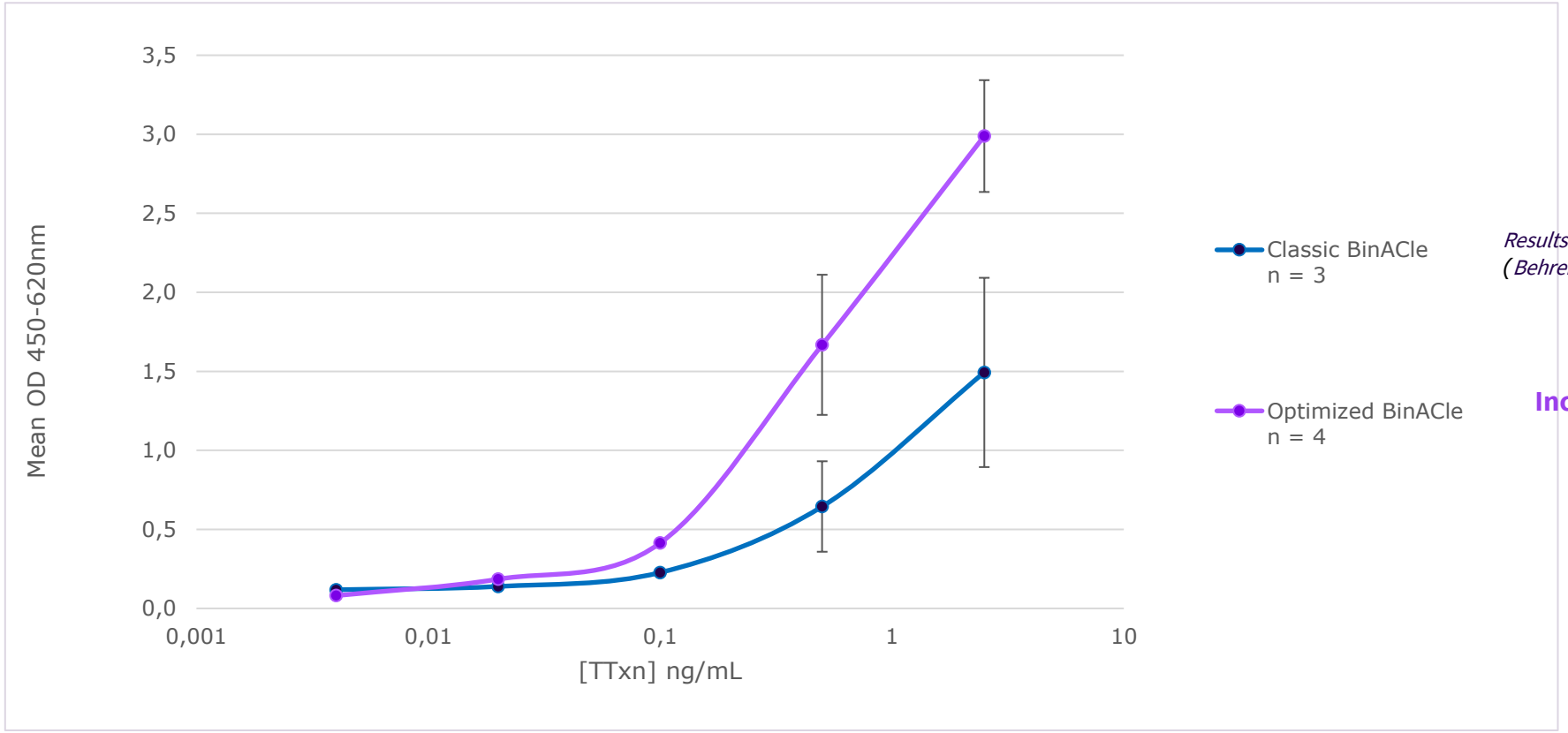


✓ 3 cleaved-Vamp2 specific Mabs → **Mab4** is selected

Betaine and Mab4 as suitable alternative reagents

BinACLe: Dose Response - Classic vs Optimized

Examples of dose responses of TTxn reference (without TTxd matrix) in Classic & Optimized BinACLe assays



Results comparable to the publication (Behrensdorf-Nicol et al., 2010).

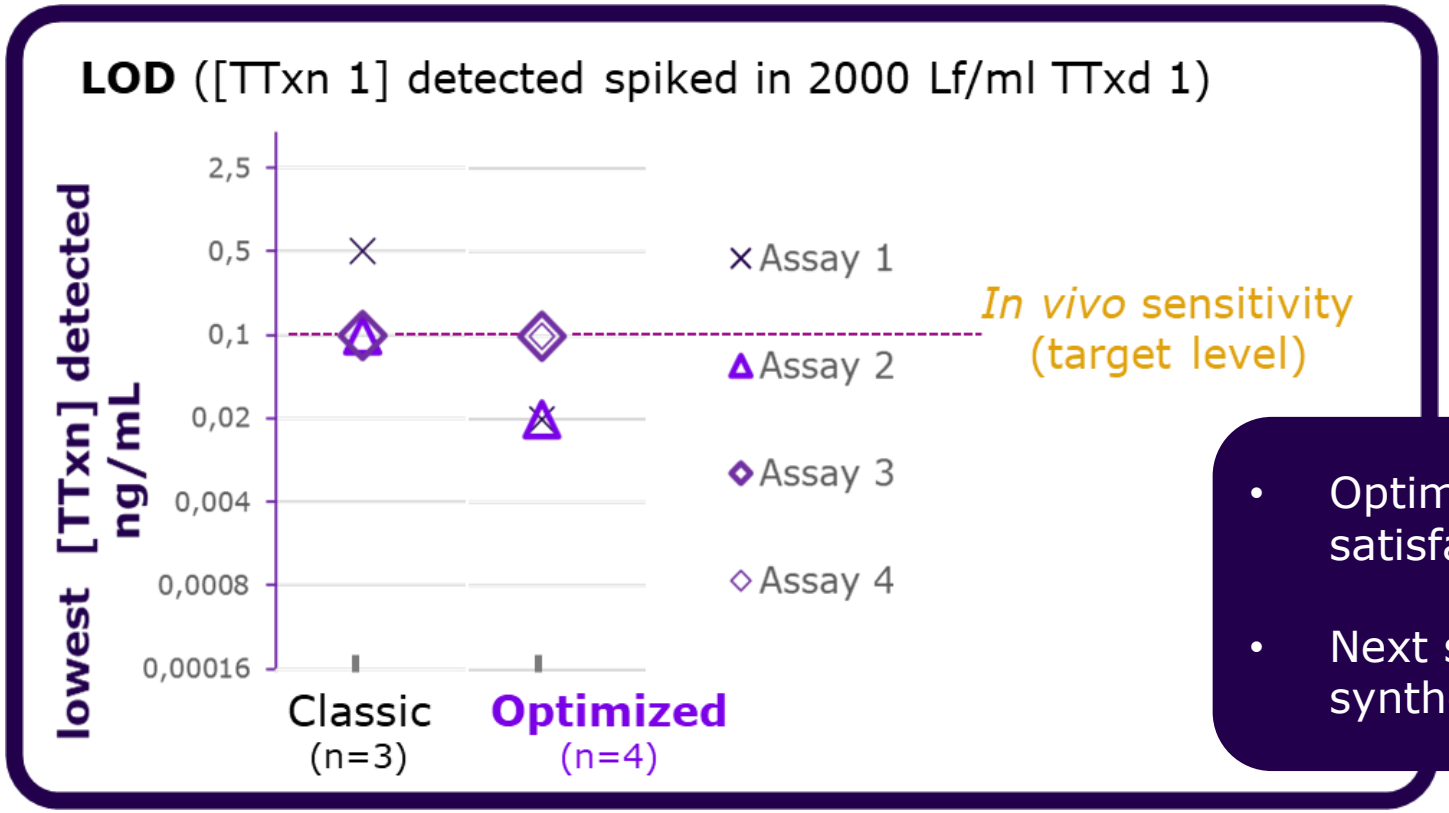
Increased dynamic range

Signals and sensitivity : Optimized > Classic

BinACLe : Performances Data

Process 1

Repeated determination of LOD for Classic & optimized BinACLe (in representative *in vivo* testing matrix, i.e. 2000 Lf/ml TTxd)



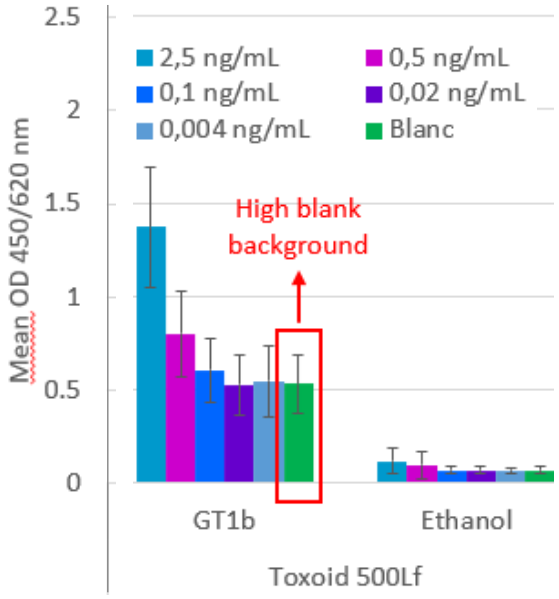
- Optimized BinACLe : satisfactory sensitivity
- Next steps : evaluation of synthetic GT1b

The LOD was estimated using a cut-off based method. ***6 Wells**
For each assay → Cut-Off = Mean (Signal Matrix Without TTxn*) + 3.3 × SD (Signal Matrix Without TTxn*)
The LOD = the lowest TTxn concentration for which at least 80 % of absorbance values(*) were above the cut-off value.

BinACLe: Performances Data

Process 2

Performance of Sanofi optimized method for Toxoid 2 (in representative *in vivo* testing matrix, i.e. 500 Lf/mL)



Proportion of Absorbance Values above Cut-off (GT1B)	
[TTxn] ng/ml	Toxoid 500 Lf
2.5	100%
0.5	11%
0.1	0%
0.02	0%
0.004	0%

LOD (ng/mL)	
Method	**Toxoid 500 Lf**
Optimized	**2.5 ng/ml**

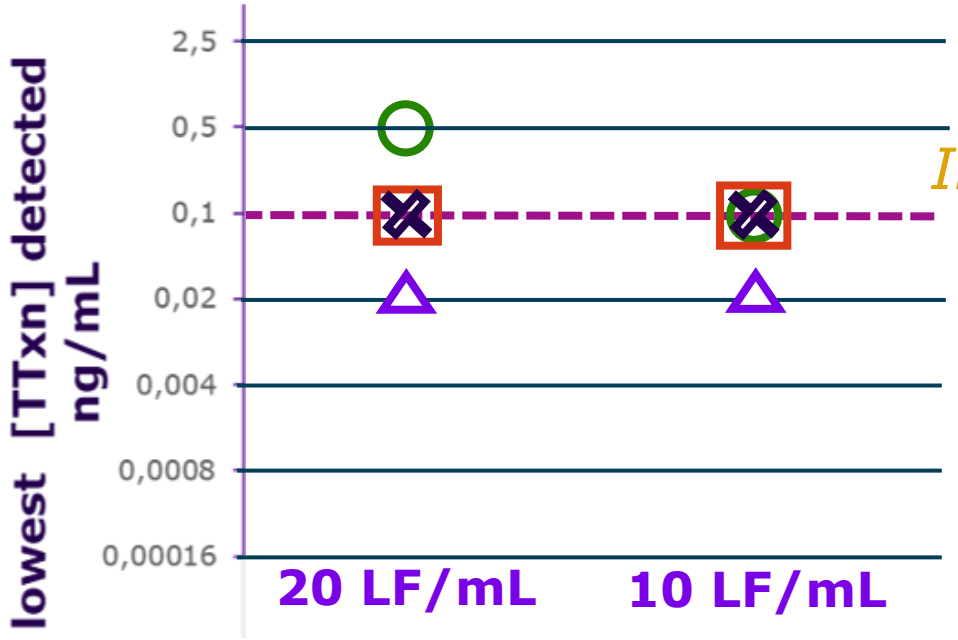
- High background of Tetanus toxoid at 500 Lf/mL impacting the LOD.
- Use the same strategy as EDQM collaborative studies: Test at a final conc. of 20 Lf/mL (for maximum of 10 Lf/SHD).
- Maximum conc. of Toxoid 2 vaccine DP is 10 Lf/mL → Product could be tested up to 10 Lf/mL.

The LOD was estimated using a cut-off based method.
For each assay → Cut-Off = Mean (Signal Matrix Without TTxn) + 3.3 × SD (Signal Matrix Without TTxn)
The LOD = the lowest TTxn concentration for which at least 80 % of absorbance values(*) were above the cut-off value.

BinACLe: Performances Data

Process 2

Repeated determination of LOD for optimized BinACLe with Toxoid 2 at 20 Lf/mL and 10 Lf/mL, spiked with TTxn 1 or TTxn 2



- Satisfactory sensitivity when spiked with TTxn 2 at 10 Lf/mL
- Low sensitivity for 20 Lf/mL when spiked with TTxn 2

In vivo sensitivity (target level)

- Intermediate TTxn 1 spike (20 Lf/mL n=6) (10 Lf/mL n=6)
- Drug substance TTxn 1 spike (20 Lf/mL n=12) (10 Lf/mL n=12)
- Intermediate TTxn 2 spike (20 Lf/mL n=21) (10 Lf/mL n=6)
- Drug substance TTxn 2 spike (20 Lf/mL n=18) (10 Lf/mL n=9)

The LOD was estimated using a cut-off based method.
For each assay → Cut-Off = Mean (Signal Matrix Without TTxn) + 3.3 × SD (Signal Matrix Without TTxn)
The LOD = the lowest TTxn concentration for which at least 80 % of absorbance values(*) were above the cut-off value.

Remaining challenges for next steps

Assay challenges

GT1b replacement

Availability of **critical reagents (mAbs)**

Toxin Reference standard choice and **acceptance criteria setting**

Implementation challenges

Comparative validation to current ***in vivo*** specific toxicity test

Introduction of the assay in **Compendia** (Pharmacopoeias, Guidelines)

No existing **Toxin Reference Standard** (WHO/EDQM)

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BINACLE

Requirements for industrialization of the assay

Shahjahan SHAID
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gsk.com

This work was sponsored by GlaxoSmithKline Biologicals SA. Shahjahan Shaid is an employee of the GSK group of companies.

3R improve the quality of science by addressing the animal welfare

Our 3Rs[1] strategy of replacement, reduction and refinement is a science-led, ethical framework that we use to guide us in our work with animals. Where animal research is conducted or commissioned, we are advancing the 3Rs (Replacement, Reduction, Refinement) and seeking ways to minimise animal use and reduce the impact on the animals.

Replace = Information gathered with a different approach

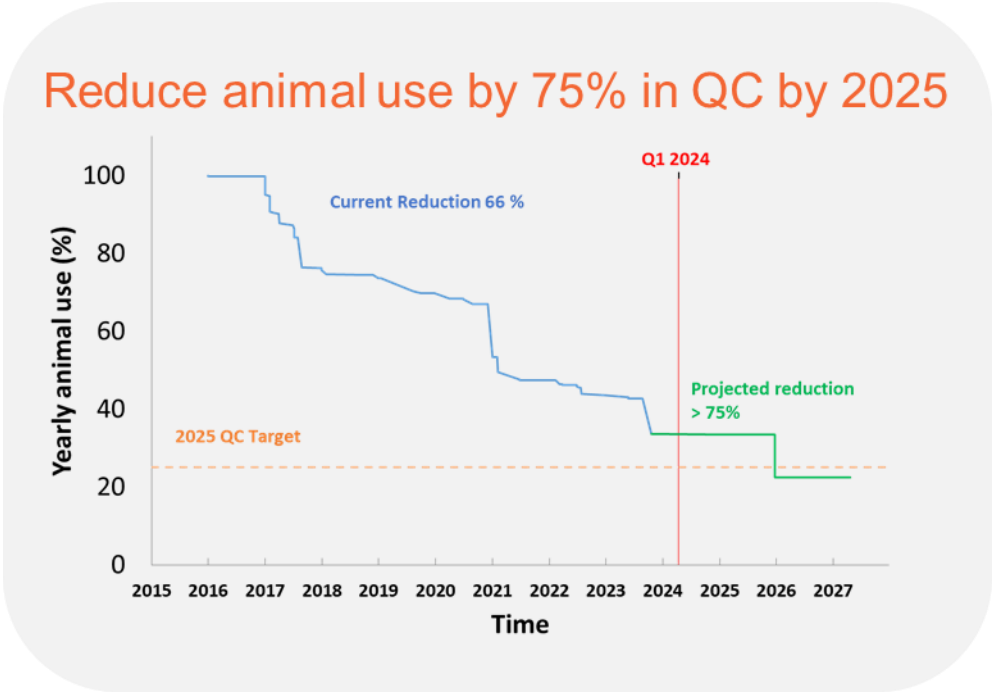
Accelerating the development and use of models and tools, based on the latest science and technologies, to address important scientific questions without the use of animals

Reduce = Animals versus Information

Appropriately designed & analyzed animal experiments that are robust and reproducible, & truly added to the knowledge base

Refinement = Less pain and distress, increased welfare

Advancing research into animal welfare by exploiting the latest in vivo technologies and by improving understanding of the impact of welfare on scientific outcomes

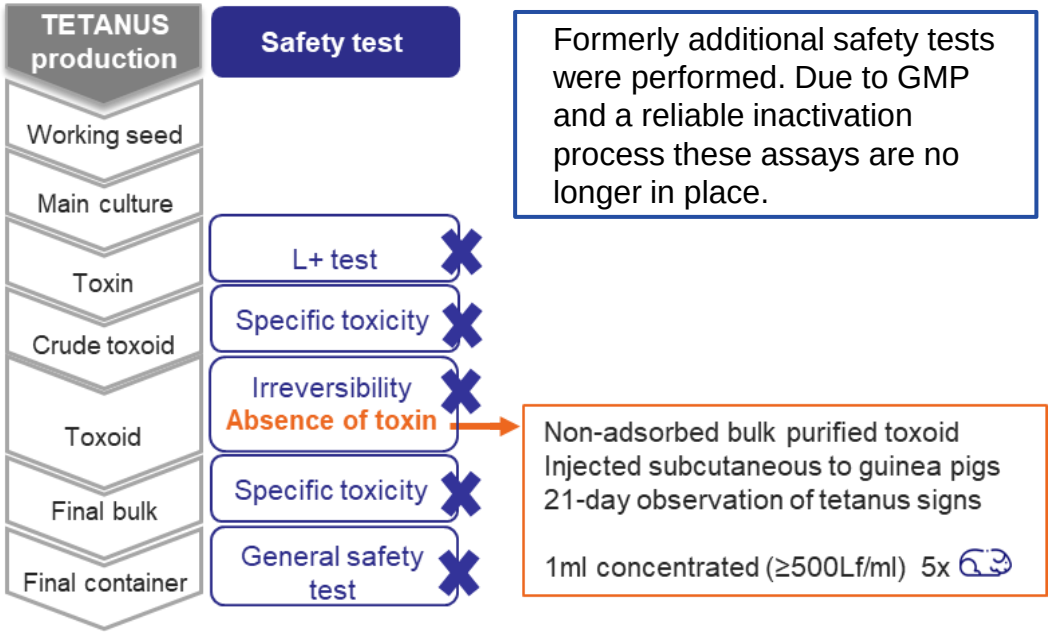


[1] The 3Rs principles were developed in 1959 with the standard definitions recently expanded by the NC3Rs

The number of animal tests has been reduced for Tetanus Vaccines

Absence of Toxin is the last in vivo safety assay and the last assay to analyse toxicity

Simplified Manufacturing process



The *in vivo* « Absence of toxin » test [*Ph. Eur*. 0452] is currently **the only** test available to determine the Absence of Tetanus Neurotoxins (TeNT) in Tetanus toxoid vaccines prior to release.

A replacement, as the BINACLE, will therefore need to ensure the same level of sensitivity as the in vivo assay to prove the detoxification process was successfully completed.

The BSP136 performed very well in GSK laboratories with a positive feedback from technicians regarding the execution. No issues were encountered.

To industrialize the assay for cGMP release several elements were investigated by GSK in 2024 to understand if there is a secure supply of required reagents.

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.

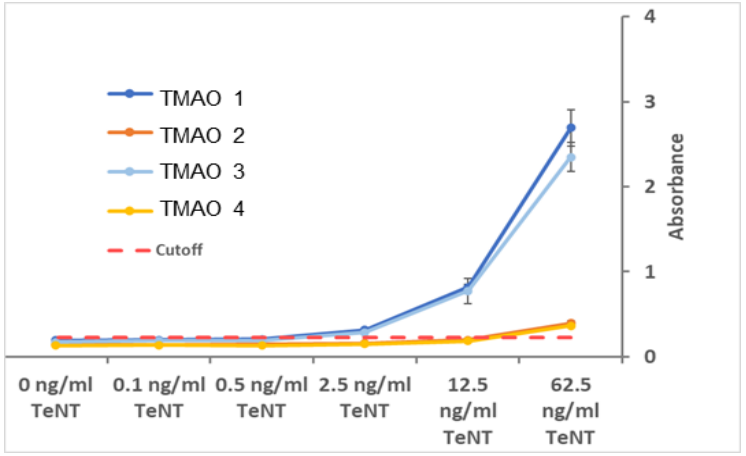
GSK

Critical reagent TMAO shows batch to batch variability

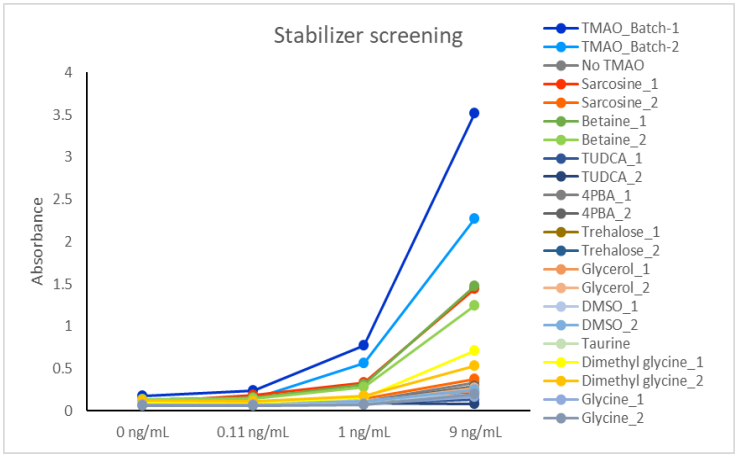
TMAO is required to increase the sensitivity but might need a repalcement

Reagents	Function	Issue	Proposal
TMAO Trimethylamine oxide	• Osmolyte that improves sensitivity of the BINACLE	• Functionality depending on batch to batch variability • GMP supplier not in place	• Identify supplier with reliable Quality and GMP • Replace

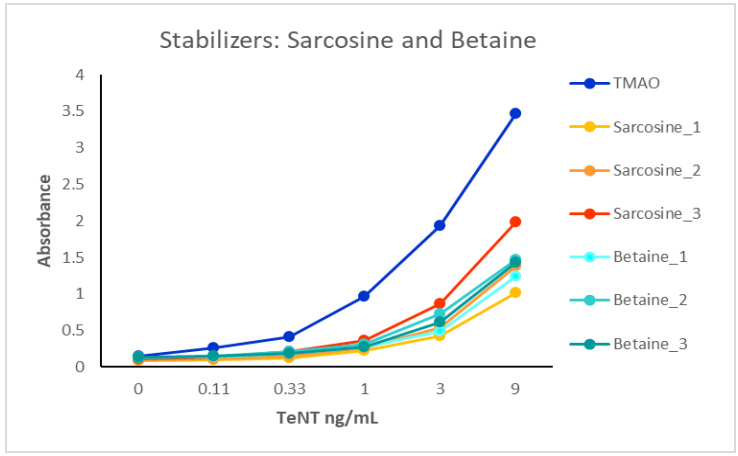
Suitable and unsuitable unsuitable batch of TMAO



Several alternatives were tested at different concentrations



Sarcosine or Betaine could be a potential replacement for TMAO in BINACLE

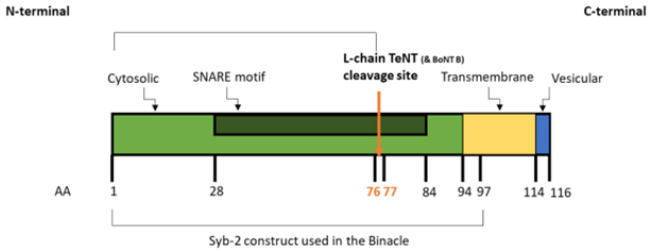


Antibodies as the core of the assay are not commercially available

Sera protocol or even better a monoclonal antibodies need to be generated

Reagents	Function	Issue	Proposal
Anti-synaptobrevin antibody sera	• Quantify specifically cleaved Synaptobrevin with a high Sensitivity (LOD)	• Protocol 3rd party property • Long term supply not guaranteed	• Develop similar procedure • Replace with monoclonal
Anti-synaptobrevin monoclonal antibody	• Quantify specifically cleaved Synaptobrevin with a high Sensitivity (LOD)	• Commercially not available • Chance of similar sensitivity and specificity as in vivo	• Share available reagents with user and a neutral 3rd party

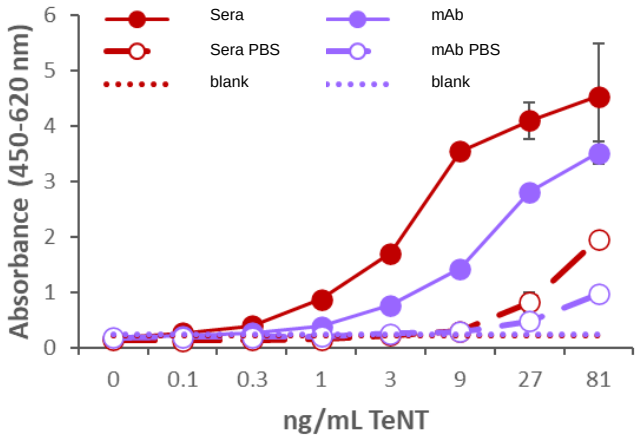
Target antigen is the cleaved site



Access to a specific and sensitive antibody

- **Sera generated by 3rd party**
- **mAb**
  - **Purchase**
  - **Animal Free**
  - **Hybridoma**

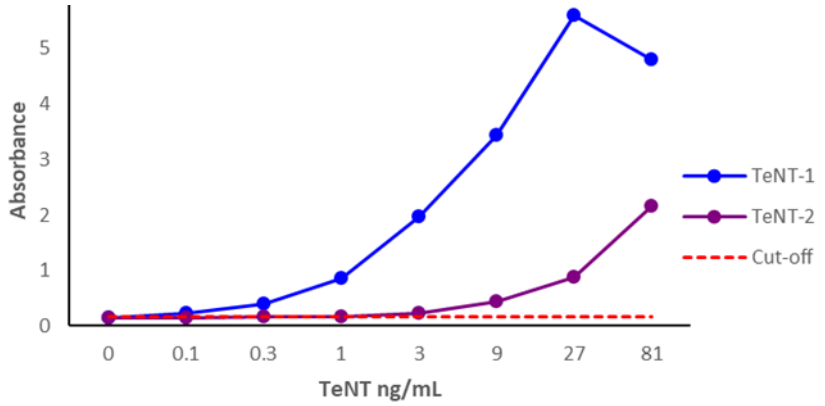
mAb compared to PEI pAb with ListLabs Toxin

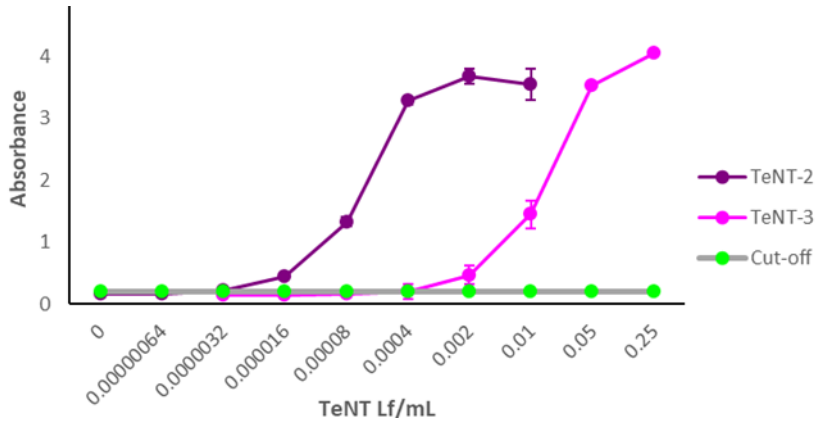


Standardization of other critical reagents as antigen and controls

An alignement on standardization will increase the uptake of the BINACLE

Reagents	Function	Issue	Proposal
Recombinant synaptobrevin-2	• Critical analyte targeted by TeNT	• Not available from GMP supplier (Quality and supply) • Expression system not standardized (LOD impact)	• Identify a reliable supplier • Agree on a preferred expression system e.g. E.Coli
TeNT Positive Control	• Positive control	• Functionality vary between suppliers (matrix effect tbc) • GMP supplier not in place	• Identify a standard or develop in house standards





The LOD in BINACLE differs between the 2 tested commercial lots.

Will regulators outside of Europe accept a replacement?

What will be required to prove superiority of the BINACLE over the *in vivo*?

- To validate the BINACLE for the specific toxoid and linked manufacturing process it will be crucial to demonstrate that:
  - *Matrix impact (potential interference of toxoids from the routine production process)*
  - *Determine the Limit of detection of a reference toxin in the manufacturing matrix.*
  - *Determine pass/fail criteria's e.g. sensitivity of spike recovery, limit for tested batches*

- The gold standard today is the *in vivo* assay while the Limit of detection (LOD) has not been determined for toxins from the routine process taking into account matrix impact, as it is not required as a compendial method.

GSK

Today the BINACLE can`t replace the in vivo assay for GMP release

The assay performance cannot ensure reproducible results due to the critical reagents. This would put at risk the release and availability of Tetanus Vaccines.

Tomorrow it could be ready: A collaboration of Vaccine manufacturer led by a neutral party could overcome this and bring the BINACLE assay into GMP routine.

Collaboration

- A reliable and GMP confirm supply of critical reagents such as antibodies and TMAO/or surrogates
- An agreement of the need to standardize or not reference material such as TeNT
  - Instead of having several vaccine suppliers overcoming this issue. Reagents and standards could be shared to harmonize those elements where feasible
- An understanding of the acceptance criteria's from other authorities.
  - Instead of several manufacturers generating data to their best knowledge, a workshop with veterinarian and human regulators expressing their requirements would streamline and accelerate a replacement

GSK

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