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

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  - **the AVAILABILITY**
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INTRODUCTION: TETANUS NEUROTOXIN AND THE BINACLE ASSAY

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Tetanus neurotoxin

Tetanus neurotoxin (TeNT) produced by *Clostridium tetani*

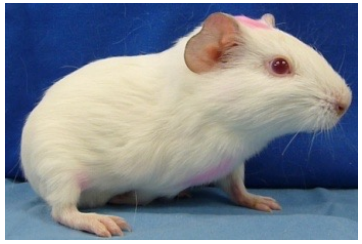
- Targets inhibitory interneurons → **Muscle spasms**, asphyxiation
- Extremely **potent** (lethal dose for humans + many animals: **~1-10 ng/kg** body weight)

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



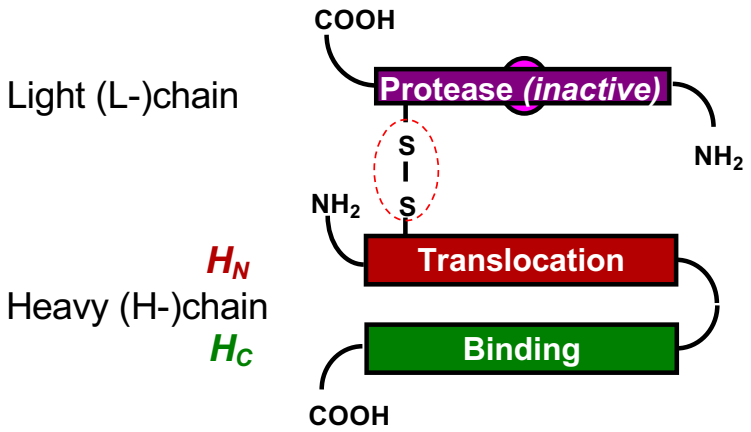
Reasons for developing *in vitro* methods

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)

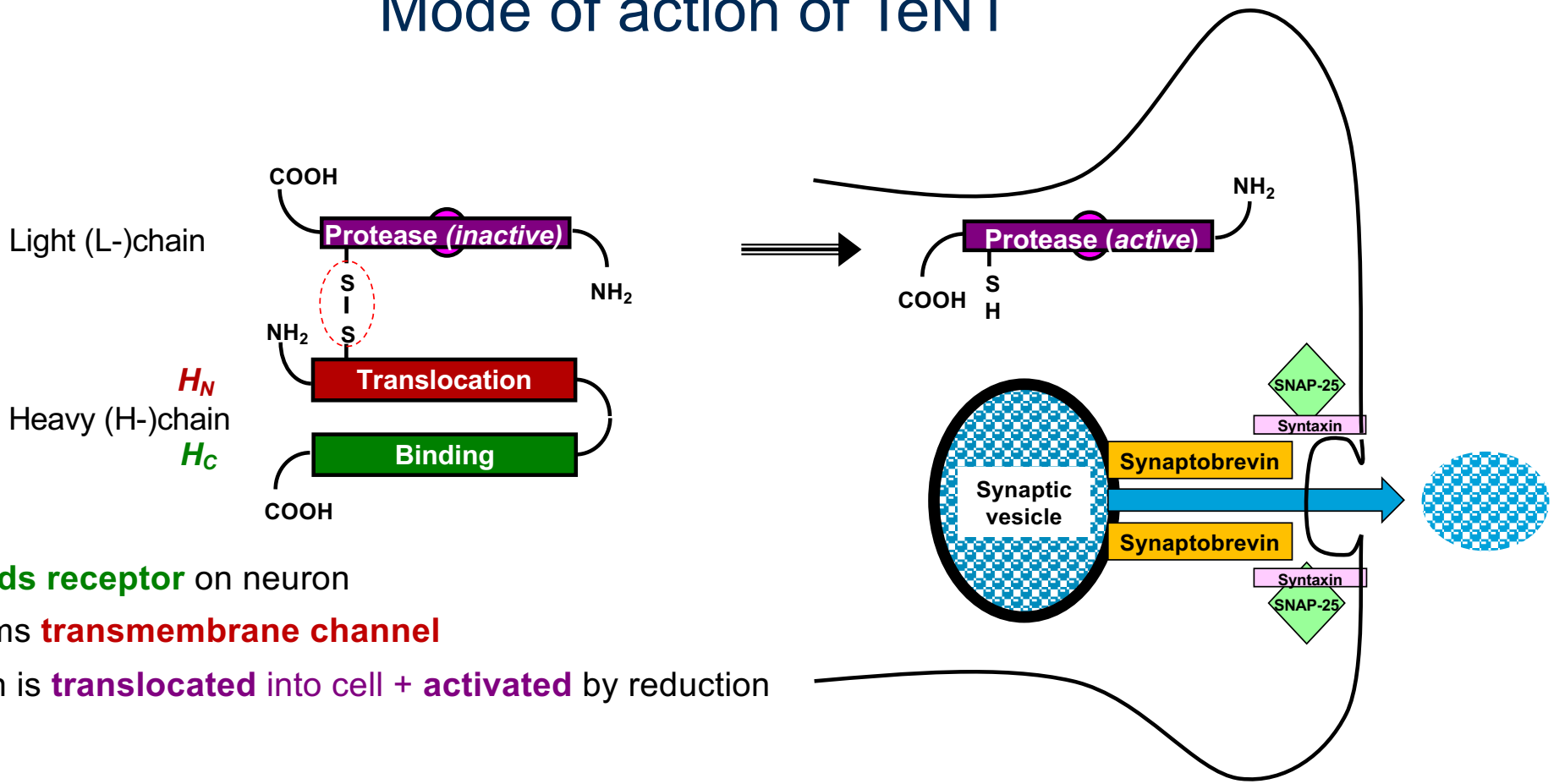
→ Replacement by *in vitro* method preferable

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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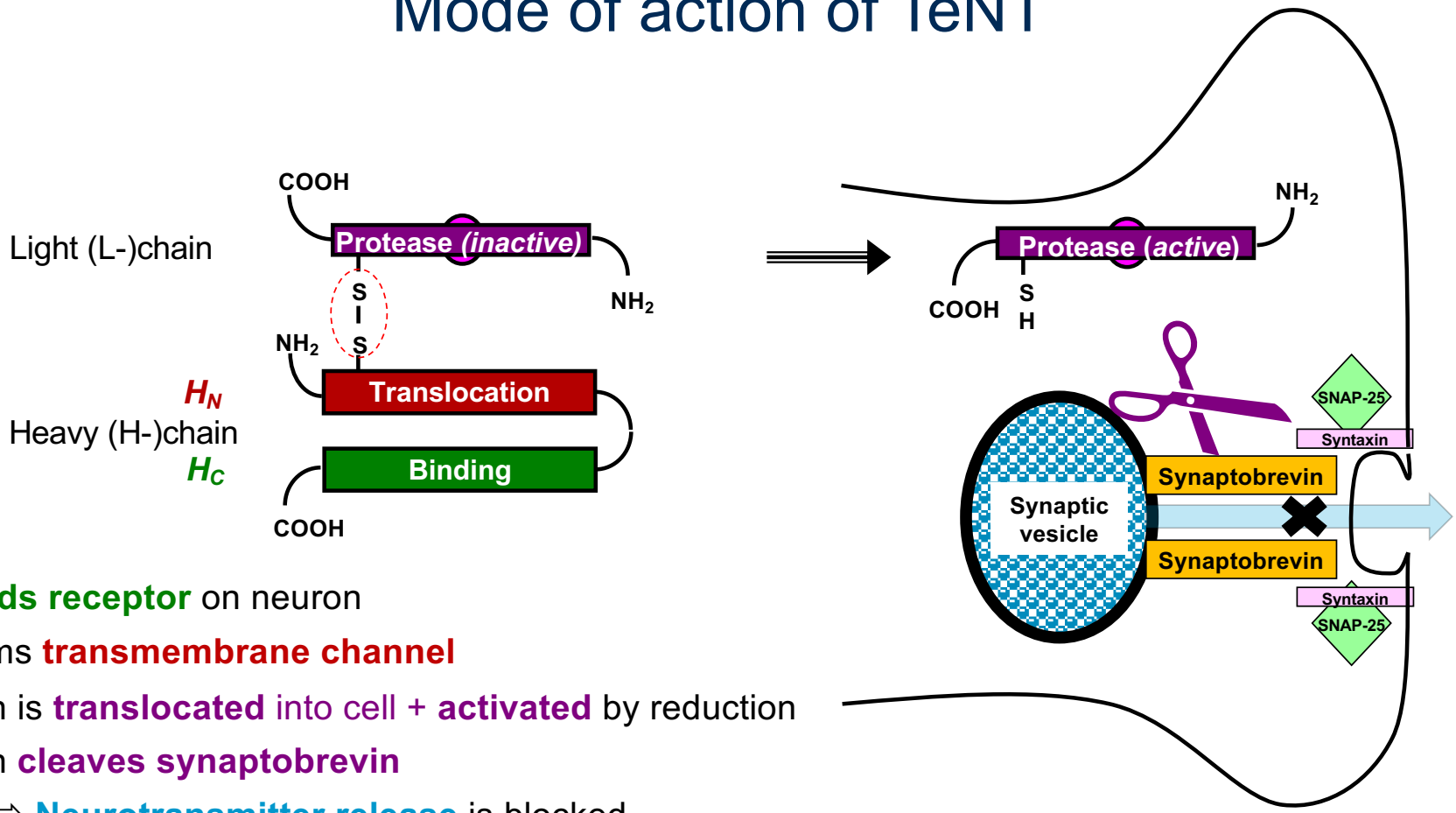
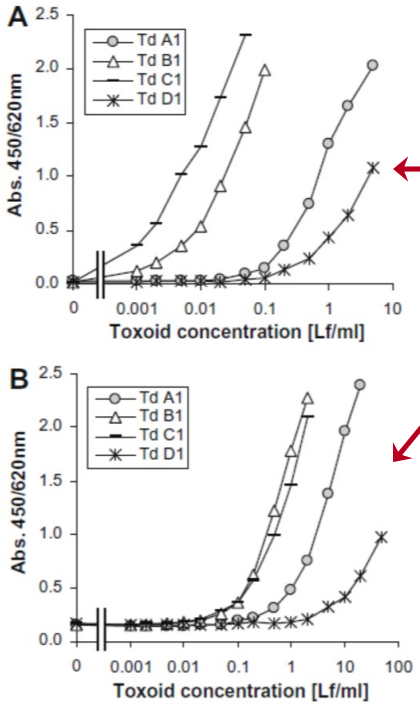
3. L-chain is **translocated** into cell + **activated** by reduction

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]

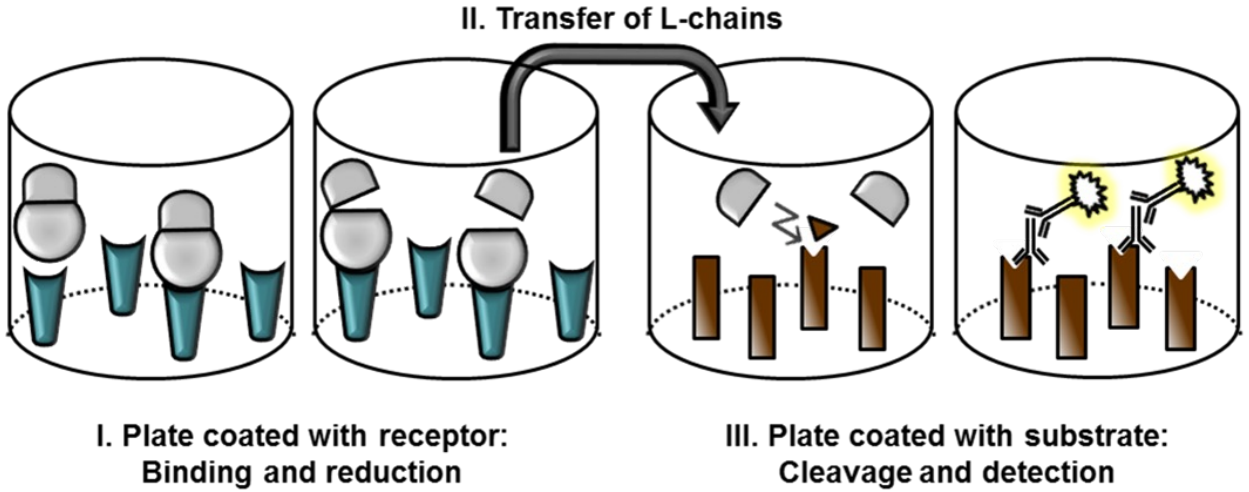
Toxoids (various manufacturers) tested in

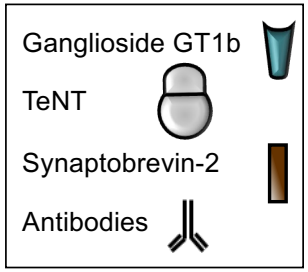
(A) **receptor-binding assay**
(B) assay for **synaptobrevin-cleaving** activity

- All toxoids showed **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



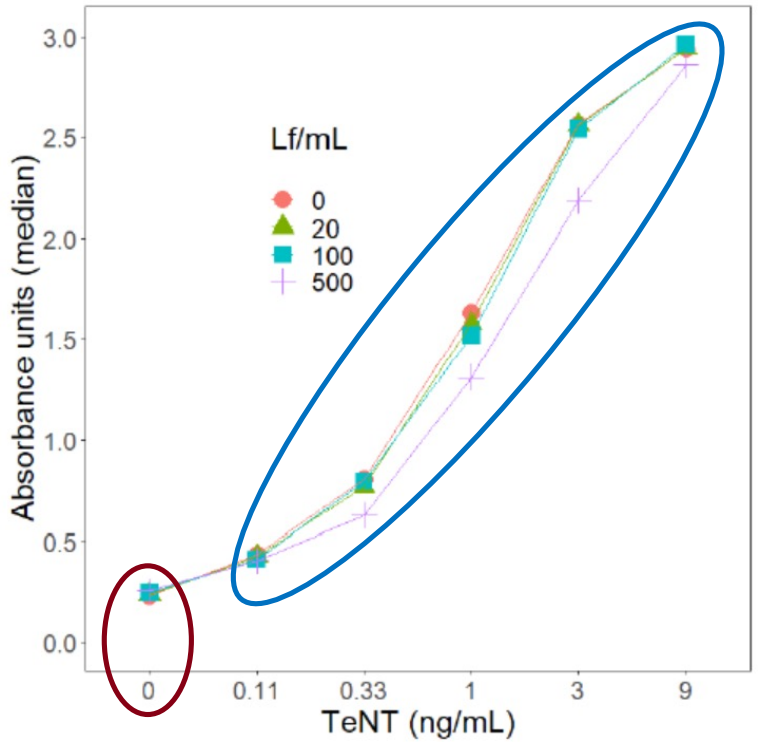


[Figure adapted from: Behrensdorf-Nicol H, Weisser K, Krämer B (2015), ALTEX 32:41-46]

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

BINACLE assay:

- Non-spiked toxoid: Signals did not exceed blank value even when tested at high concentration (500 Lf/ml)
- Toxoid spiked with TeNT: Clear dose-response-relationship, sensitive TeNT detection

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

(**Note:** Some toxoids may not be suitable for BINACLE testing; toxoids from some sources induce elevated background signals.)

COLLABORATIVE STUDY BSP 136

Collaborative study for TeNT BINACLE assay

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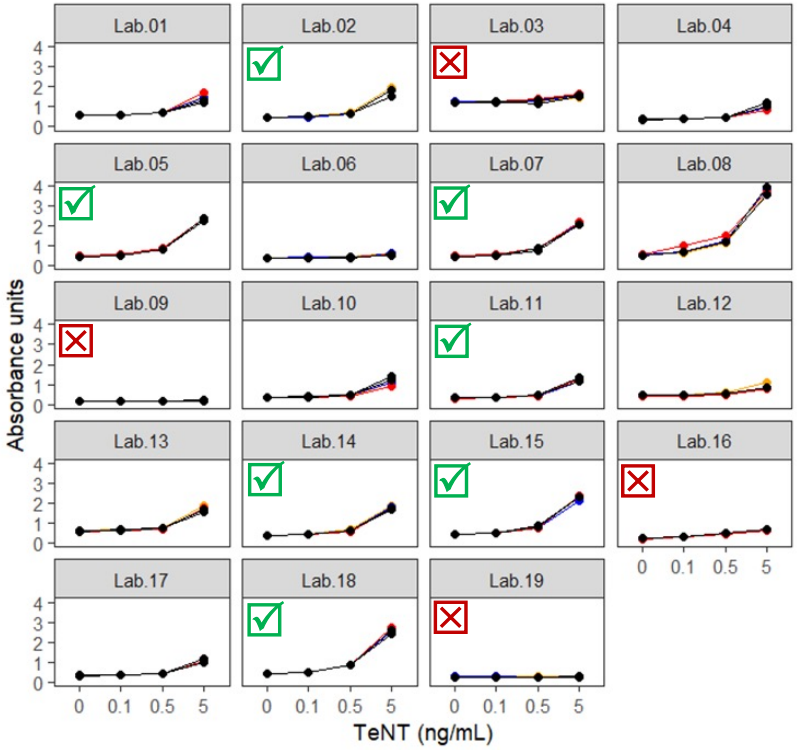
International collaborative study (BSP 136):

- Organised by EDQM (European Directorate for the Quality of Medicines & HealthCare)
- In the context of the Biological Standardisation Programme
- Participants: Vaccine manufacturers, national control laboratories
- Detailed assay protocol + critical reagents were supplied to participants
- Test samples: TeNT diluted in tetanus toxoids (to mimic insufficiently inactivated toxoid)
- Each participant performed 3 to 4 BINACLE tests

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

Collaborative study, part 1



[Figure from: Behrensdorf-Nicol H, Krämer B, Le Tallec D, Sinitskaya N, Behr-Gross ME (2024) Pharmeur Bio Sci Notes 2024:127-161]

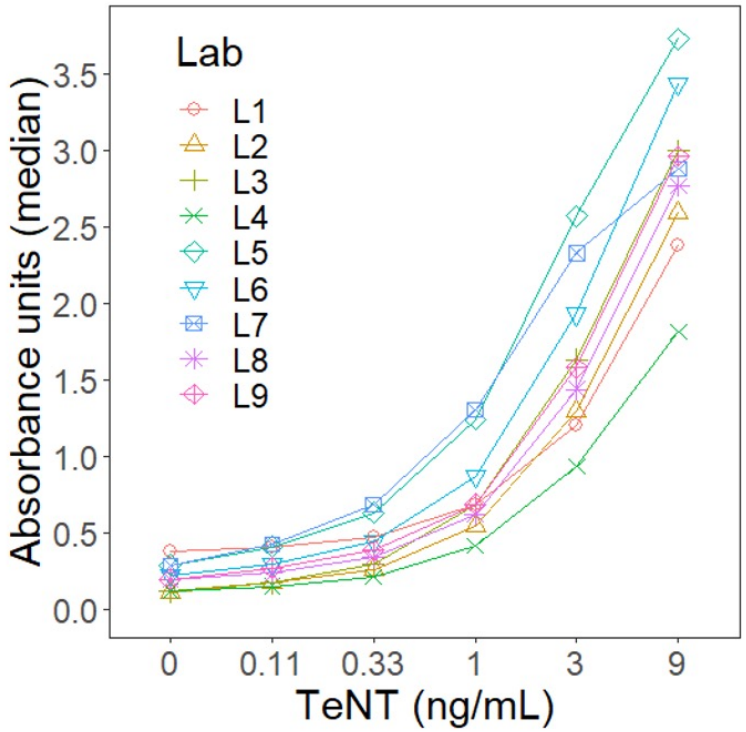
Results of study part 1 (19 participants):

- Most participants obtained clear dose-response curves
- But: **High variability**
  - 7 laboratories: Sensitive TeNT detection (≤0.5 ng/mL) ☑
  - 4 laboratories: Unable to detect even 5 ng/mL TeNT ☒

→ Based on these data, **improvements were introduced** to reduce variability and enhance standardisation
  - Protocol optimisation
  - More pre-qualified reagents

→ **Improved BINACLE assay** tested in **study part 2**

Collaborative study, part 2



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

Results of study part 2 (9 participants):

- All participants obtained clear dose-response curves
- Detection limit calculated by cut-off-based method:

  cut-off = mean blank value + 3.3 x standard deviation

  – all laboratories were able to detect 0.33 ng/mL TeNT
  – 5 laboratories were able to detect 0.11 ng/mL TeNT

→ **Detection limit of BINACLE assay:** in **same range** as the estimated detection limit of the **animal test**

Collaborative study, part 2 (continued)

Variability* (indicated as geometric coefficients of variation):

Intra-laboratory variability (average)	12 %
Inter-laboratory variability	4 %
Reproducibility (intra- + inter-laboratory variability)	13 %

(* calculated by ANOVA based on relative potency values determined for TeNT in toxoid relative to TeNT in buffer)

→ Variability is **similar to commonly reported values for immunochemical assays**

CONCLUSION AND OUTLOOK

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Conclusion

- Optimised **BINACLE assay** allows **reliable detection of active TeNT**
  - **Transferability:** All participants successfully performed the method •
  - **Detection limit:** Equivalent to estimated detection limit of *in vivo* test •
  - **Variability:** Acceptable for a multi-step assay •
- **Note:** BINACLE assay **may not be applicable to all toxoids**, toxoids from some sources induce high background signals
- For suitable tetanus toxoids, the **BINACLE assay** may represent a **good alternative to the safety test in guinea pigs**

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Outlook

- Discussions about inclusion of the BINACLE assay into the **European Pharmacopoeia** as alternative to the guinea pig test for 'Absence of tetanus toxin' are underway

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

  Key point: It must be shown that **non-compliant bulks** are **reliably detected**
  → to make sure that the **switch to *in vitro* testing** has no negative impact, but rather **contributes to high product quality + safety**

- **Webinar on TeNT BINACLE** assay: **12 November 2024** (organised by EDQM)
  → for more information, see www.edqm.eu, Events & training

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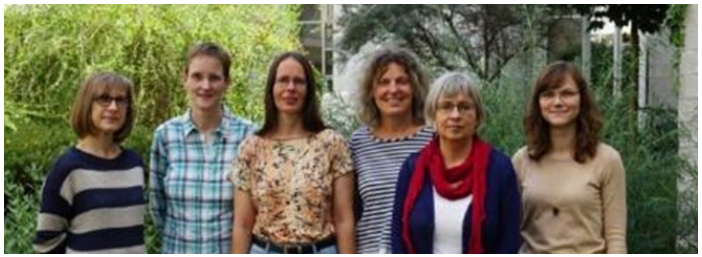

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AnimalfreeResearch

DFG Deutsche Forschungsgemeinschaft German Research Foundation

THANK YOU VERY MUCH

FOR YOUR ATTENTION