3R success stories: Rabies and Leptospira case studies

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Introduction

Replacement of *in vivo* test by *in vitro* methods is an on going and long lasting conversation between Animal Health industry and Health Authorities.

- In the EU, this has involved long term collaboration with regulatory agencies and official medicines control laboratories (OMCLs)

- Following EU approvals companies have succeeded in replacing the *in vivo* test in many international markets also

- The following presentation will walk through two examples for 2 different antigens for which the finished product testing have been replaced from an *in vivo* method to an *in vitro* method. Both examples show the application of the consistency approach which is described in EP monograph 5.2.14.

RABIES:

REPLACEMENT OF THE CURRENT *IN VIVO* RELEASE POTENCY/IDENTITY TEST ON FINISHED PRODUCT BY AN ALTERNATIVE *IN VITRO* ELISA

Regulatory Procedure

Replacement of the current *in vivo* release potency/identity (serology) test on finished product by an alternative *in vitro* ELISA assay measuring glycoprotein (GP) content for all the concerned products.

Zoetis products involved:

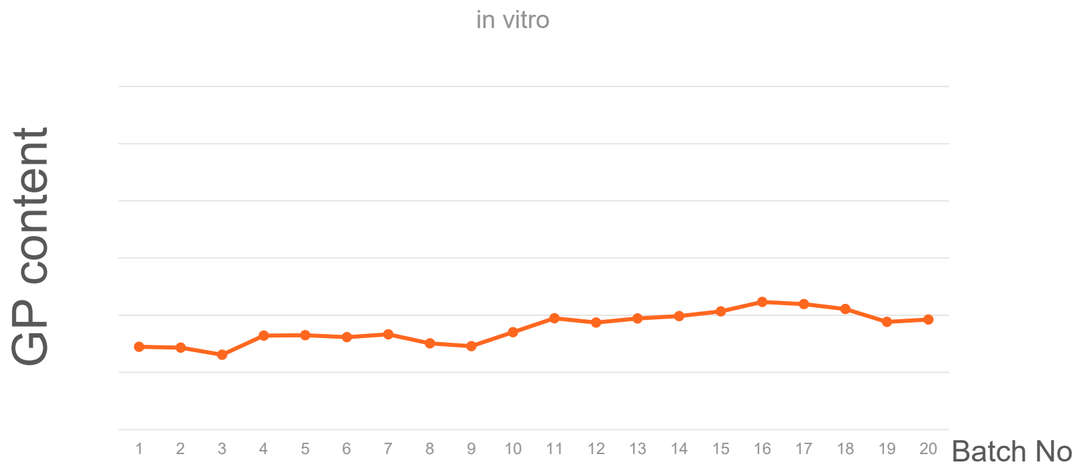
- **Multivalent containing Rabies**
- **Monovalent Rabies**

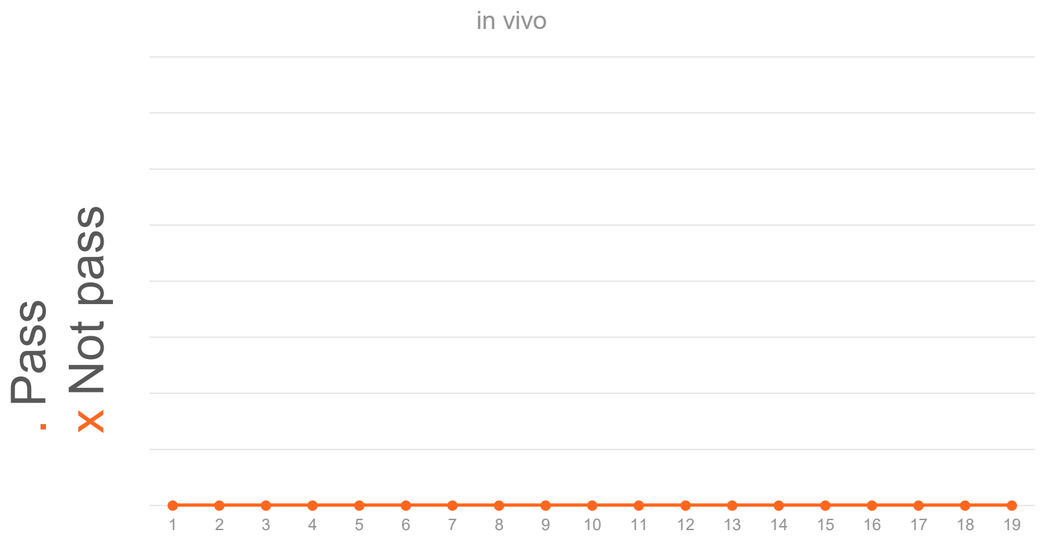
Data package at submission

- Description of the methodology and reagents used
- Validation document of the method according to VICH guidelines 1 and 2
- Qualification of an internal reference standard against a Biological Reference Preparation
- Consistency data of consecutive recently manufactured batches
- Stability studies under normal conditions of different batches
- Forced degradation studies

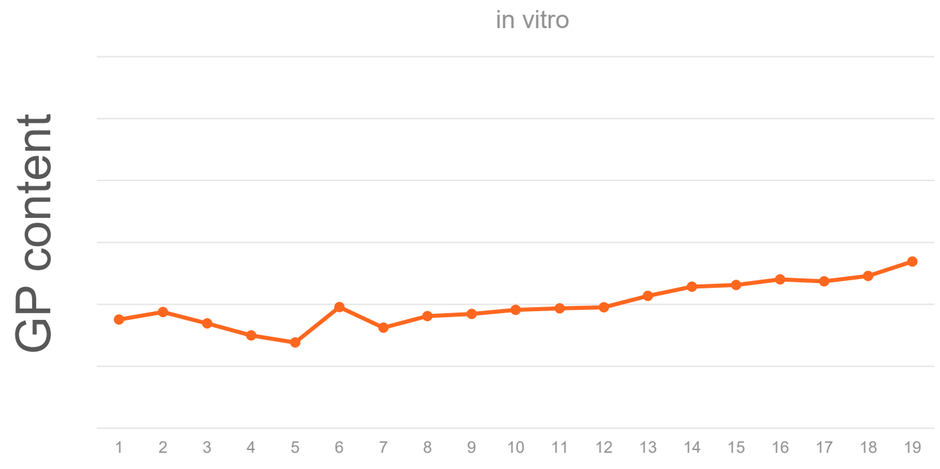
Consistency data:

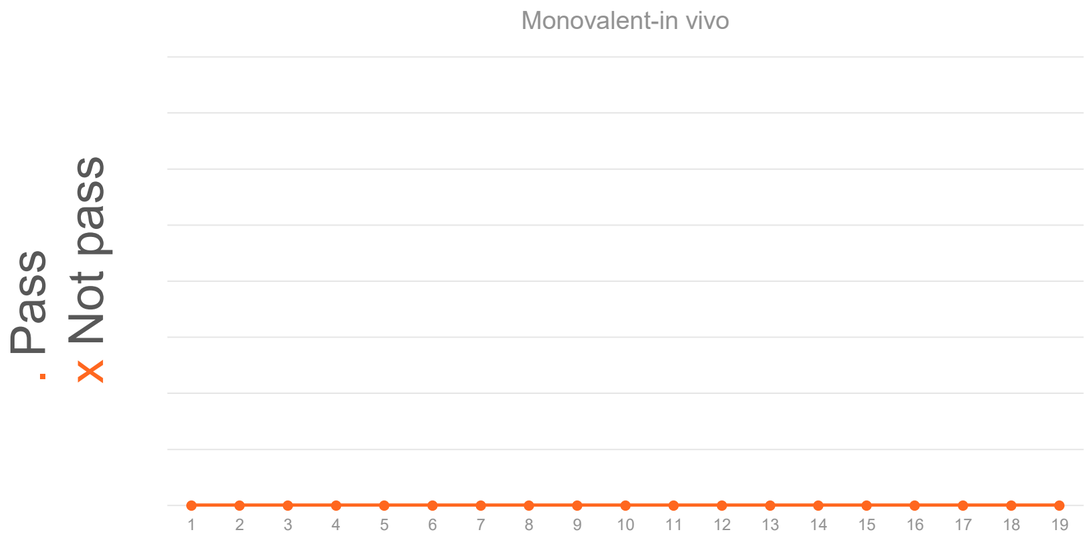
Multivalent data





Monovalent data





Rationale for setting the specifications at release:

- Stability and forced degradation studies

Overall, the results show a variable decrease in GP content from batch to batch ranging from **nearly none** up to **40%** for the lots evaluated.

Vaccine Lot	No treatment		95°C treatment		65°C treatment		42°C treatment	
	Serology	ELISA	Serology	ELISA	Serology	ELISA	Serology	ELISA
#1	Pass	Pass	Not Pass	Not Pass	Not Pass	Not Pass	Pass	Pass
#2	Pass	Pass	Not Pass	Not Pass	Pass	Pass	Pass	Pass
#3	Pass	Pass	Not Pass	Not Pass	Not Pass	Not Pass	Not Pass	Not Pass

ELISA Vs Serology

The ELISA has demonstrated a higher discriminatory power than the current serology release test to detect different degrees of degraded antigen. 100% qualitative correlation was found using both methods

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Rationale for setting the specifications:

As is often the case and in line with **Ph. Eur. 5.2.14 (Substitution of in vivo method(s) by in vitro method(s) for the quality control of vaccines)**, **when is not possible to show full agreement between the *in vitro* and *in vivo* methods** due to the low discriminating power and/or high variability of the *in vivo* assay, **a consistency approach is the appropriate way forward for setting specifications for the new ELISA assay**.

This approach is further justified based on the following points:

- ELISA data available for a number of commercial batches
- The ELISA assay is able to detect antigen degradation under thermic stress conditions and it is stability indicating
- The rabies containing vaccines involved in this procedure are well established products which have been demonstrated to be safe and efficacious for many years in the market

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Rationale for setting the specifications:

- **Maximum content**

Glycoprotein content from a safety study performed using a batch with a known concentration of GP and Consistency data

- **Minimum content**

Glycoprotein content from consistency batches and stability data. Additional data from an efficacy study with a known concertation of GP

Proposed specifications:

at Release

Proposed spec MIN GP content (IU/mL)	Proposed spec MAX GP content (IU/mL)
Min release based on consistency and stability data	Max content tested on safety study

at EOSL

Proposed spec in GP content at EOSL (IU/mL)
≥ 5.0

MAJOR QUESTION

Questions:

It is essential that the specific monoclonal antibody recognises only the trimeric form of the antigen as it is the key epitope. Therefore, more information with regard to the antibody used in the kit is needed and should be provided.

Information available provided by the Kit developers. Neutralising Antibody

It should be demonstrated that the mAb used in the kit detects only forms of the rabies virus glycoprotein (RABV-GP) that are inducing neutralising antibodies to the same extent as native trimeric RABV-GP.

- Literature
- Demonstration of ability of the mAb to detect the relevant glycoprotein conformation

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Literature-Rabies virus

The Rabies virus (RABV) contains a single-stranded negative-sense RNA genome that encodes five structural proteins:

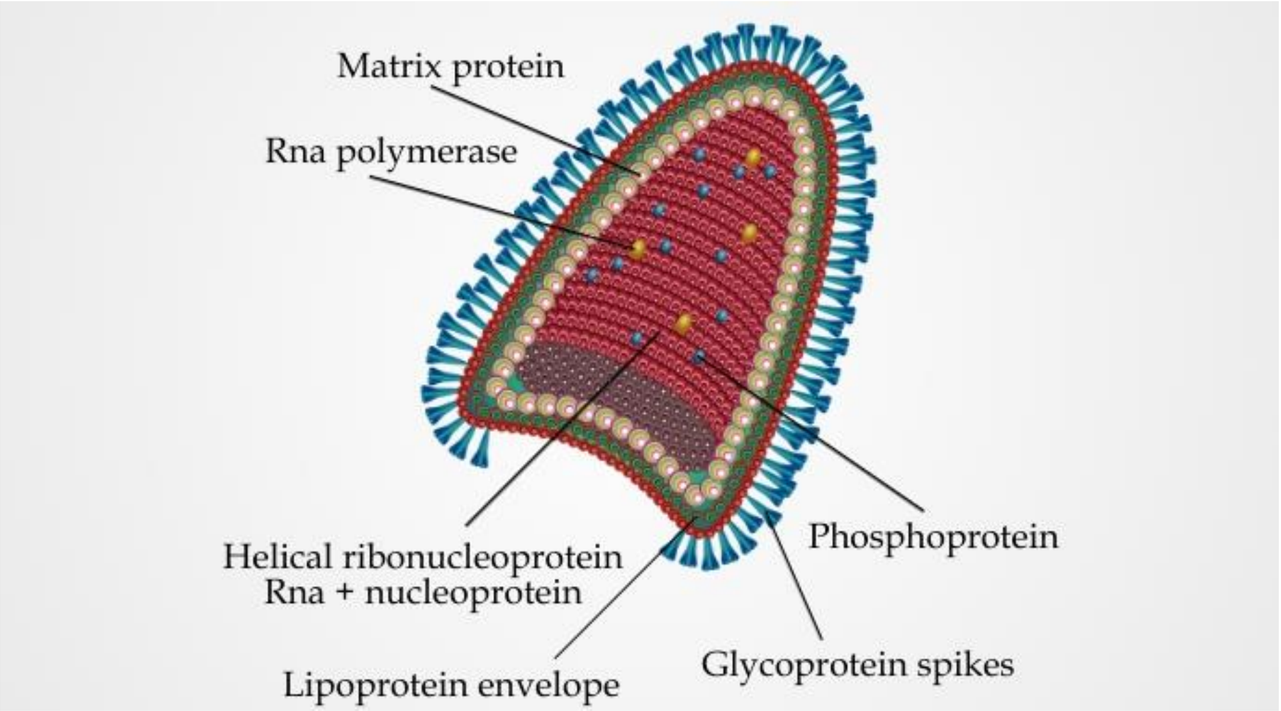
(N) Nucleoprotein

(M) matrix protein

(G) Glycoprotein

(P) Phosphoprotein

(L) RNA-dependent RNA polymerase



(Yang et al., 2020) and picture from https://www.freejpg.com.ar/istocksim/1026657282?s=1

Literature-Glycoprotein

The glycoprotein of RABV (RABV-GP) plays a pivotal role in the pathogenesis of the virus by **mediating both viral recognition of and attachment to cellular receptors**.

As it is the only protein present on the surface of the virus, **RABV-GP is also the major target for neutralizing antibodies.**

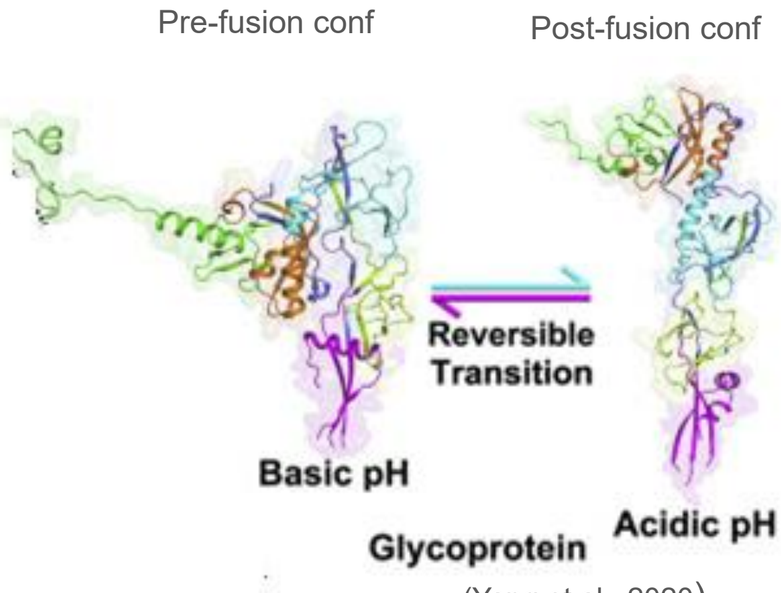
On the viral surface, RABV-G is structurally heterogeneous and **only a portion is recognizably trimeric.**

RABV-GP could transit through different conformational states and these states are proposed to exist in **a pH-dependent thermodynamic equilibrium, such that it is shifted from the pre-fusion state toward the post-fusion state as the pH decreases.**

This results in a **reversible structural transition, which distinguishes RABV-GP from other viral glycoproteins** that undergo completely irreversible transitions induced by a reduction in pH.

Such reversibility is proposed to enable RABV-GP recovery to its native pre-fusion conformation after transport through the acidic compartments of the Golgi apparatus.

The pre-fusion conformation is considered the relevant conformation to induce immunogenicity



(Yang et al., 2020)

(Gaudin et al., 1991) (Gaudin et al., 1993) (Gaudin et al.,1995) (Gaudin, 2000) (Roche and Gaudin, 2002), (Harrison, 2015) (Yang et al., 2020) (Callaway et al.; 2022)

Zoetis's approach for assay suitability:

Demonstration of the ability of the mAb to detect the relevant glycoprotein conformation

ELISA:

Sample pH	Theoretical GP content	GP content observed
Untreated	92 IU/ml	**95.97 IU/ml**
pH=6,8	92 IU/ml	**85.34 IU/ml**
pH=4,8	92 IU/ml	**21.86 IU/ml**
pH=4,3	92 IU/ml	**0.23 IU/ml**
pH=3,8	92 IU/ml	**0.59 IU/ml**
pH=3,3	92 IU/ml	**0.17 IU/ml**

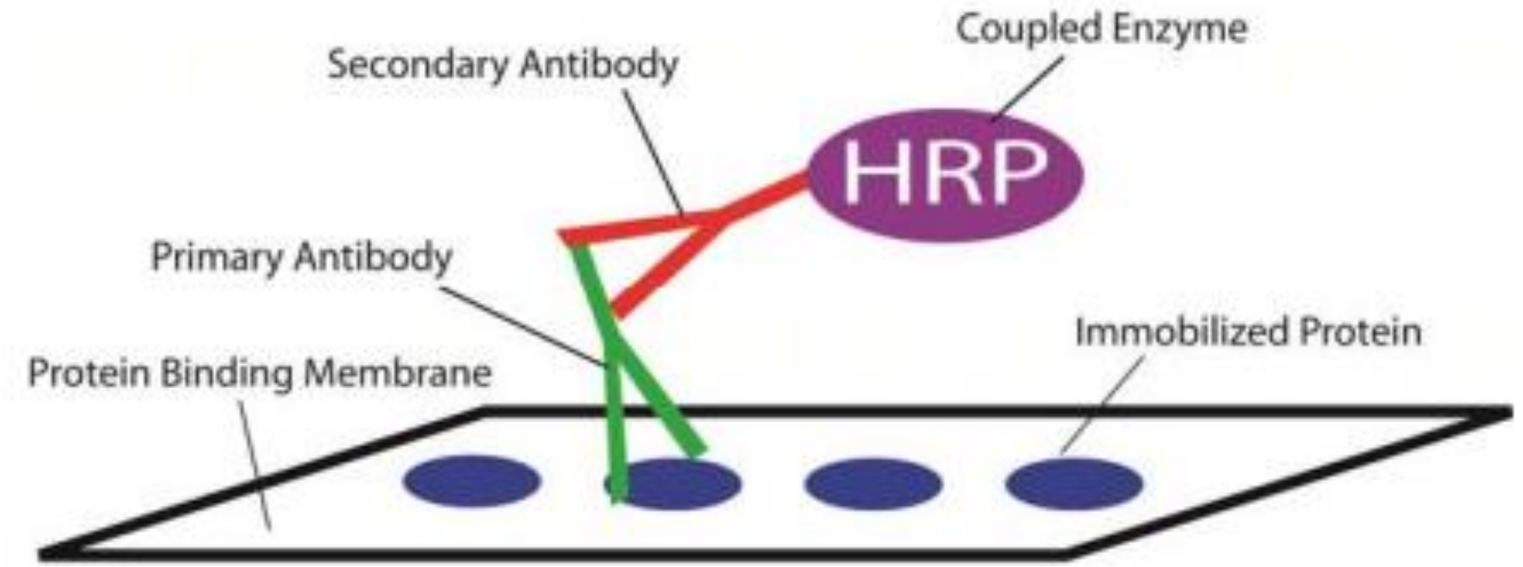
The **lower the pH** the more the equilibrium is shifted towards the **post fusion conformation**

The lower the pH the less the GP detected by the ELISA

Zoetis's approach for assay suitability:

Demonstration of the ability of the MAb to detect the relevant glycoprotein conformation

Dot Blot



Zoetis's approach for assay suitability:

Demonstration of the ability of the MAb to detect the relevant glycoprotein conformation

Dot Blot

Dilution:
1:64 1:128 1:256

1. Original Bulk
2. pH=6,8
3. pH=4,8
4. pH=4,3
5. pH=3,8
6. pH=3,3

The lower the pH the less the GP detected by the Mab

Lessons learned:

- Importance of forced degradation studies and the importance to select from the beginning the relevant conditions (pH and temperature in this case)
- Importance of consistency data
- Where possible link with efficacy/safety studies facilitates the procedure

Approvals:

Multivalent: EU + Thailand + Mozambique and South Africa

Monovalent: EU + Switzerland

LEPTOSPIRA:

REPLACEMENT OF THE CURRENT *IN VIVO* RELEASE POTENCY/IDENTITY TEST ON FINISHED PRODUCT BY AN ALTERNATIVE *IN VITRO* ELISA

Regulatory Procedure

Replacement of the current *in vivo* release potency/identity test on finished product (Hamster test) by an alternative *in vitro* ELISA assay measuring lipopolysaccharide (LPS) content for all the concerned products.

Zoetis products involved:

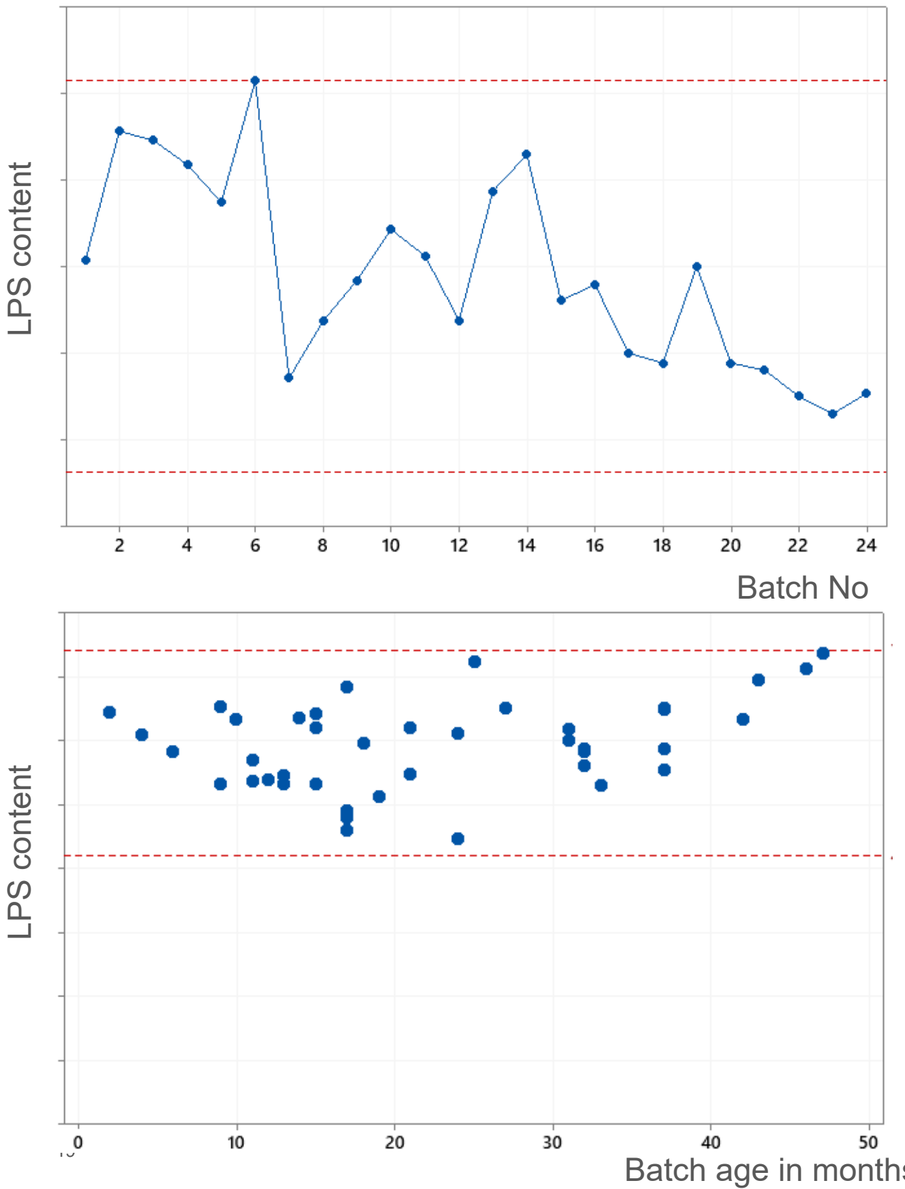
- **Multivalent vaccines (2 serovoar)**
- **Monovalent vaccines (2 serovoar)**

Data package at submission

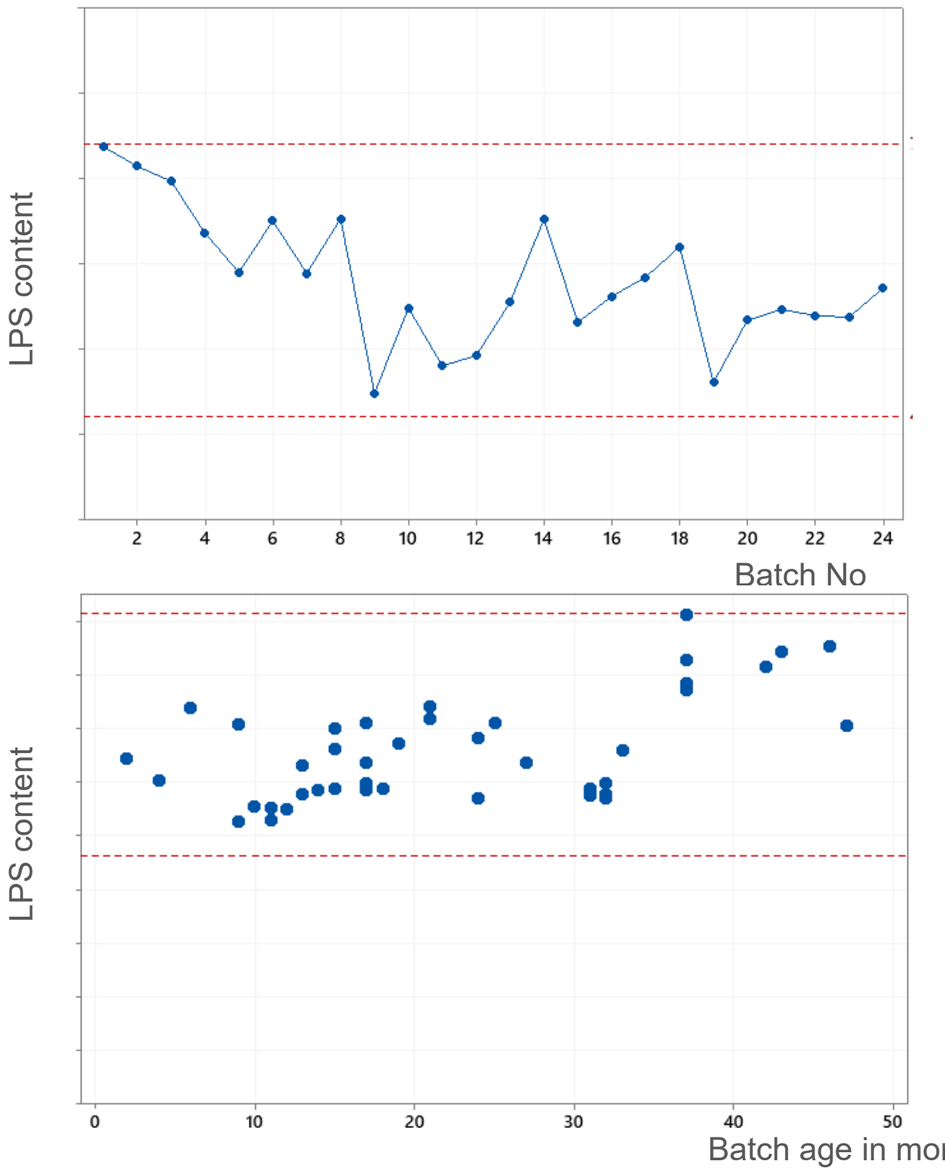
- Description of the methodology and reagents used
- Validation document of the method according to VICH guidelines 1 and 2
- Qualification of an internal reference standard
- Consistency data of consecutive recently manufactured batches
- Stability studies under normal conditions of different batches
- Forced degradation studies

Consistency and stability data

Leptospira icterohaemorrhagiae

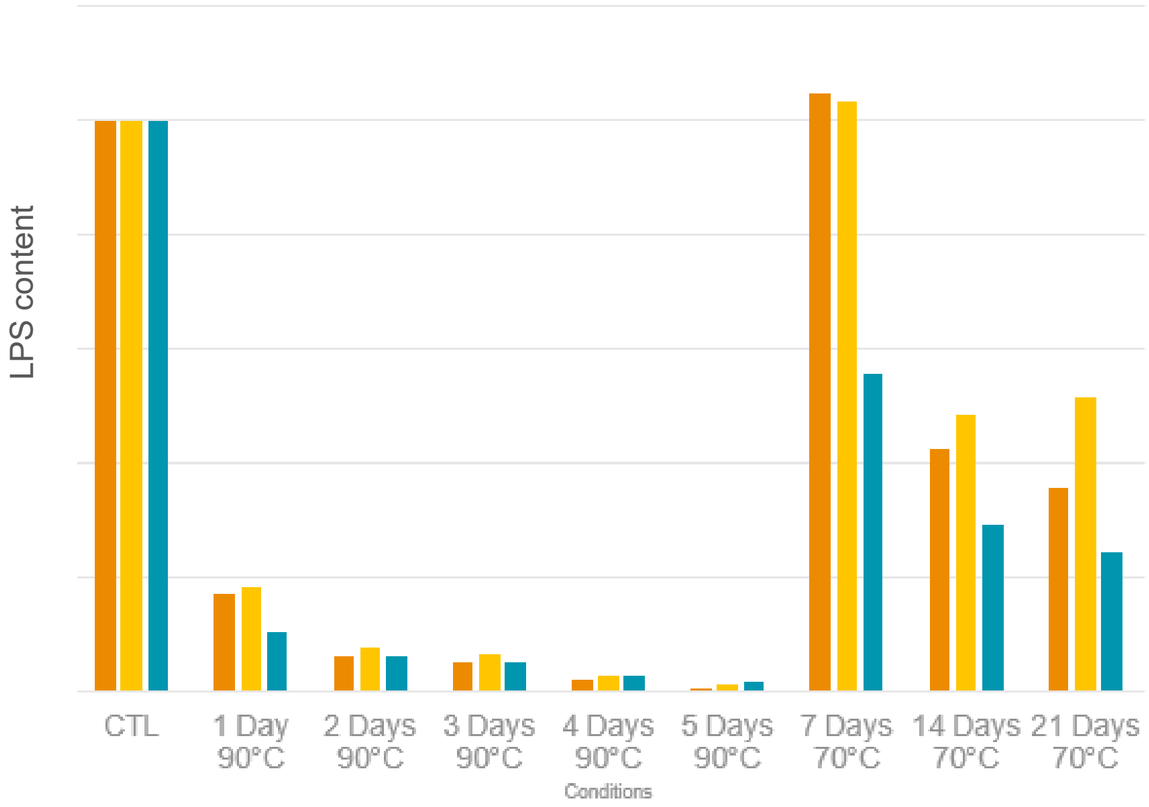


Leptospira canicola

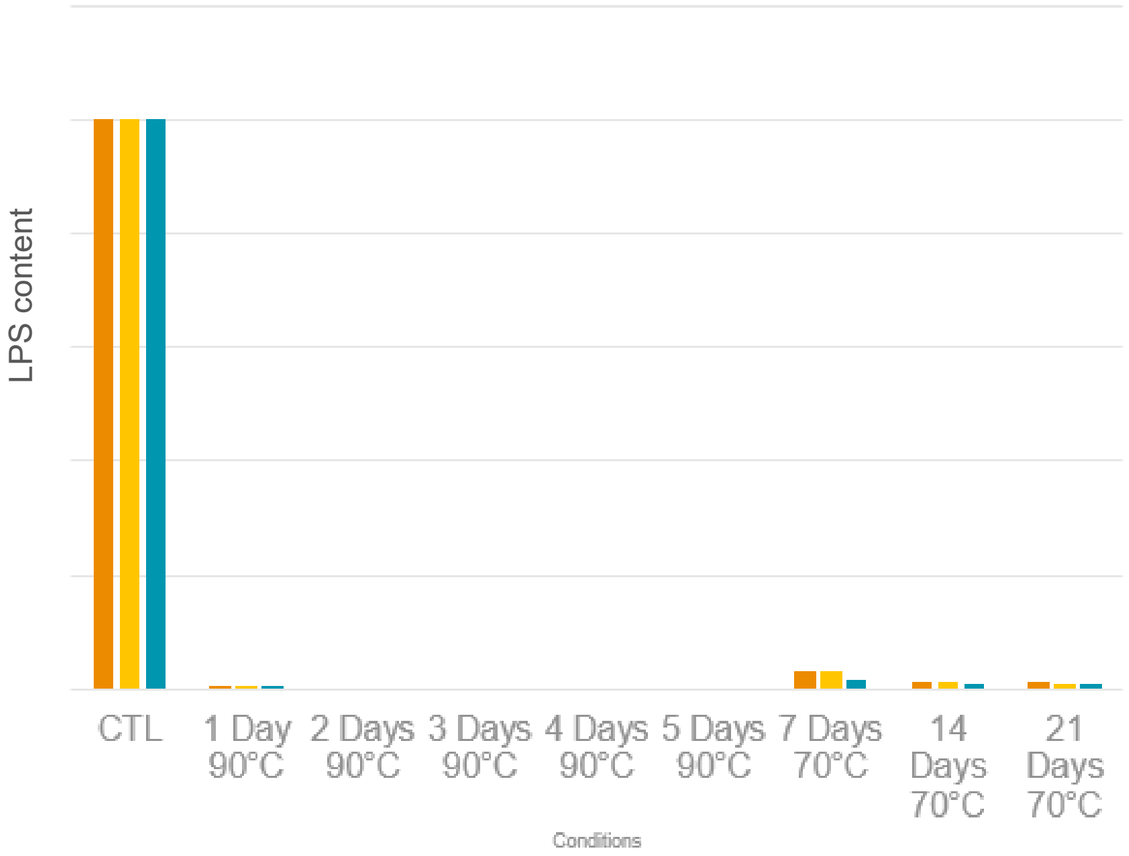


Forced degradation data

Leptospira icterohaemorrhagiae



Leptospira canicola



Rationale for setting the specifications:

- **Maximum content**

LPS content from Consistency and stability data

- **Minimum content**

LPS content from Consistency and stability data

Proposed specifications at release and end of shelf life:

Leptospira icterohaemorrhagiae

Proposed spec MIN content (RU/dose)	Proposed spec MAX content (RU/dose)
463	915

Leptospira canicola

Proposed spec MIN content (RU/dose)	Proposed spec MAX content (RU/dose)
420	740

Conclusions and key messages

Conclusions

In the 2 examples you see 3 key aspects that were critical to success were:

- Demonstrating the suitability of the method to detect an immunologically relevant antigen

- Establishing discriminatory power – ensuring the method could detect both sub-potent and not consistent batch

- Using the consistency approach to establish relevant release and end of shelf-life specifications

Key Messages

- One single assay method may not be suitable for all products (i.e Rabies)
- No direct correlation between the *in vivo* and *in vitro* methods BUT they are consistent in their ability to ensure potency
- Specification for existing products are based on historical consistency data
  - No need to repeat challenge tests to establish specifications
  - No need to run challenge tests to establish a reference
- Using the same methods and stability data to establish both release and end of shelf-life specifications
- In all cases it does take a significant amount of time and resources to develop the method and generate the data to establish and support specification and gain regulatory approvals
  - Better product consistency
  - Reduced release testing time (2 days vs 3 months for potency)
  - Reduced testing costs
  - Enhance supply continuity
    - No animal use
    - Less repeat testing
    - Time (more shelf-life on product)

Thank you!