Industry success in approval of Rabies Glycoprotein ELISA methods for potency

AFSA-HealthforAnimals Webinar

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animalhealth europe
THE VOICE OF THE ANIMAL MEDICINES INDUSTRY

Introduction

Its taken over 40 years discussion but the Animal Health industry in the EU have now succeeded in replacing the NIH/Ph.Eur. *In vivo* Rabies potency test with *in vitro* immunological methods based on Glycoprotein content

- 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 the approach of BI, MSD and Zoetis with 3 different GP ELISA methods but you will see common themes throughout that are consistent with EP monograph 5.2.14 and the application of the consistency approach

Boehringer Ingelheim

RABISIN
Switch to *in-vitro* potency test

AFSA/HealthforAnimals webinars

Transition to non-animal based veterinary vaccine batch release testing. Policy and regulations theoretical aspects and case studies

8 October 2024 | Confidential

Benjamin HATAT – Regulatory Affairs Team Leader

Life forward

Agenda

- ✓ Context
- ✓ Boehringer Ingelheim ELISA method
- ✓ Establishment of limit of acceptance (release and end-of-shelf-life)
- ✓ Regulatory procedure aspects

Context

Optional short description

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Context

RABISIN is an ***I****nactivated* vaccine against rabies and is *adjuvanted* with *aluminium hydroxide*.

Since its *launch in early 80's*, all batches had been released using the *NIH test* (as per *Ph. Eur. 0451*, using the Reference preparation of rabies vaccine (*BRP*), standardized in International Units).

NIH had been *recognized worldwide for decades* as the golden standard test to assess potency of rabies vaccines, but:

- ✓ It raises critical concerns from *animal welfare* point of view and in line with the *EU Directive 2010/63* and the "***3Rs***" (reduce, refine, replace), *alternative* had to be developed and implemented,
- ✓ It remains only *semi-quantitative* when testing adjuvanted veterinary vaccines with *limited confirmation* that batches are formulated with the *right payload of rabies antigen* (dose related response only demonstrated for human rabies vaccines).

Context (…)

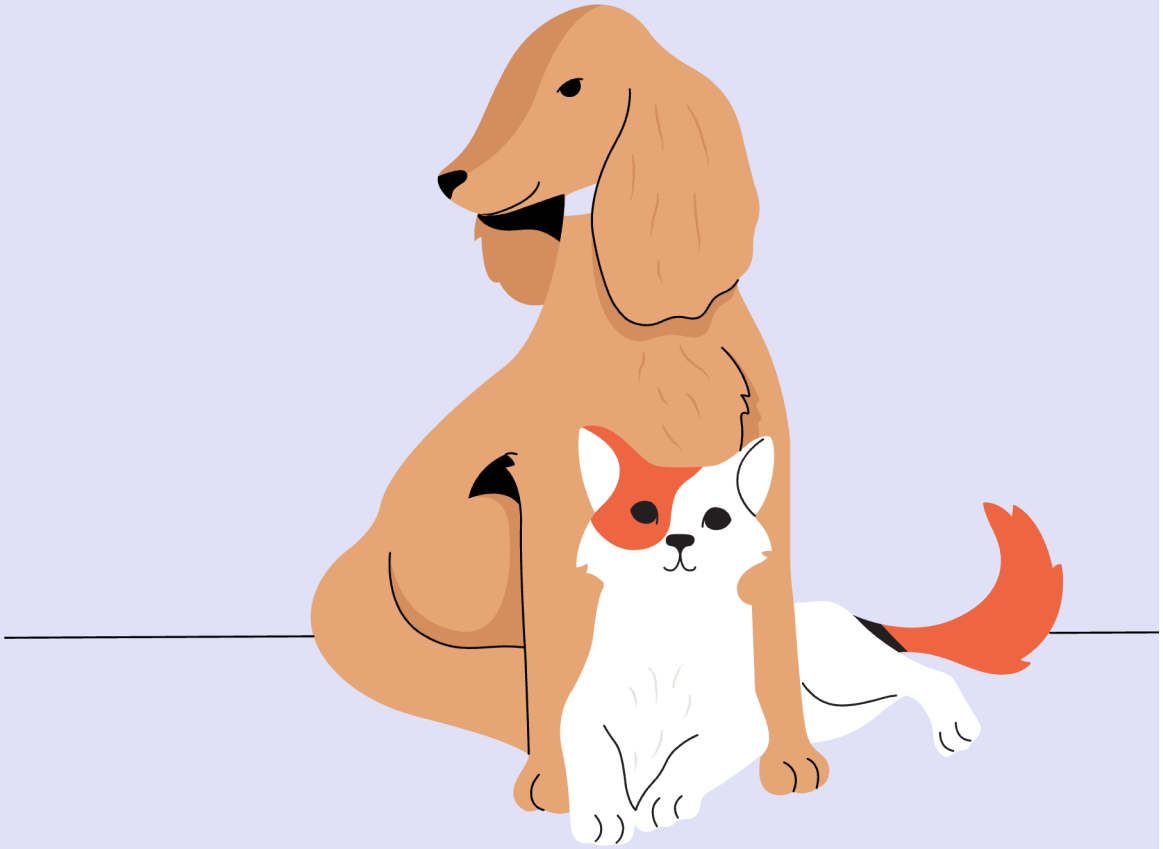
A *Serological Potency Assay*, developed and validated by *Paul Ehrlich Institute* (PEI) was tested in Europe at large scale in a *collaborative study* driven by *EDQM (2010)* and finally introduced in *Ph. Eur. 0451 (2012)* as a possible *alternative to the NIH* for routine testing of veterinary vaccine batches.

- ✓ Significant *improvement* regarding *number of animals* used and their *suffering,*
- ✓ Still *limited* from *quantitative confirmation* perspective.

MERIAL (now Boehringer Ingelheim animal Heath) developed a *full in-vitro ELISA method (2015)* and has been deploying it through *variations of marketing authorizations from 2017*.

The approach used by MERIAL and presented in the variation package complies with *Ph. Eur. 5.2.14. "Substitution of in vivo method(s) by in vitro method(s) for the quality control of vaccines" (2018*), although this latter which was still at a draft stage at that time.

Boehringer Ingelheim ELISA method



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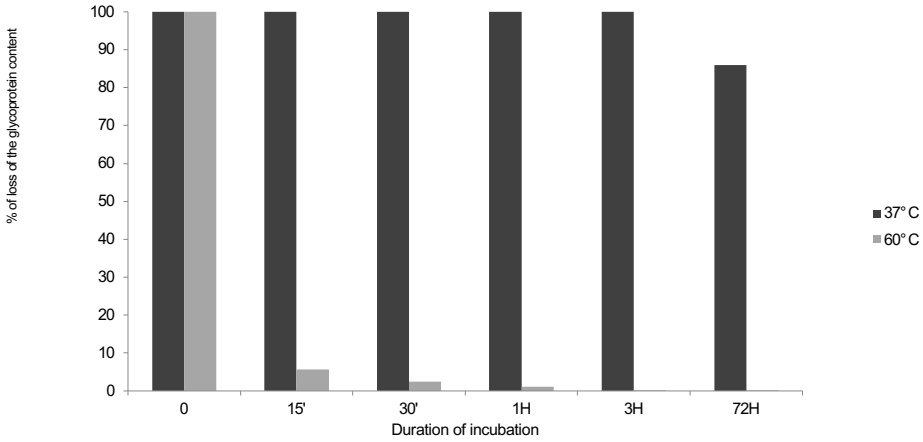
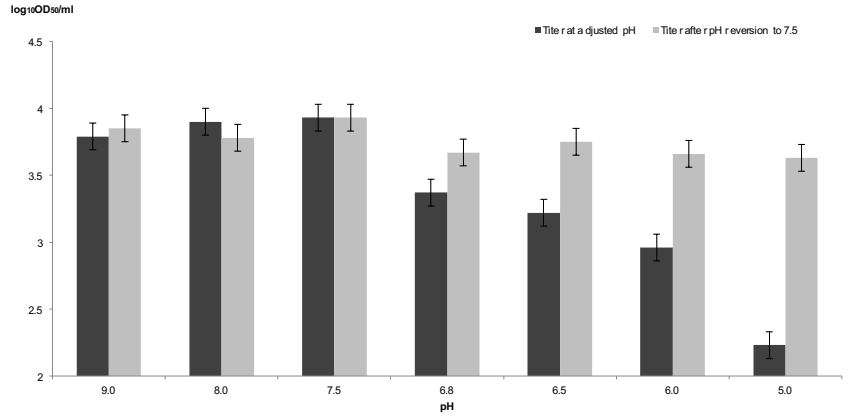
A versatile Qualitative and Quantitative ELISA (Sigoillot-Claude et al 2015)

✓ *Sandwich* ELISA using deeply *characterized monoclonal antibodies* (capture & revealing).

Effect of antigen thermal-degradation & conformation reversibility according to pH were studied (among other parameters)





=> Targets *well conformed neutralizing epitopes:*

only the *trimeric pre-fusion form of glycoprotein G* known to elicits the production of viral neutralizing antibodies is quantified.

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A versatile Qualitative and Quantitative ELISA (Sigoillot-Claude et al 2015)

✓ Ability to quantify the rabies antigen:

- During *all steps of vaccine production* (viral cultivation, downstream process, vaccine bulk formulation)
- During *all life cycle* (batch release and stability monitoring),
- In *complex environments* (presence of aluminum hydroxide adjuvant and/or other vaccine valences)

✓ Full validation performed according to *VICH GL1&2 requirements*

✓ *Best alternative to NIH*:

- Address the animal welfare concerns at its *highest level* (use of animals definitely stopped),
- More *discriminant* to confirm batch-to-batch consistency,
- *Safety benefit* for laboratory staff (no more manipulating of live rabies virus),
- *Lead-time to market significantly reduced* (especially critical for tenders).

Establishment of limit of acceptance (release & end-of-shelf-life)

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Establishment of limit of acceptance at release

✓ *ELISA results of commercial batches* of RABISIN over a period of *3 years of vaccine production* were analysed. Mean titres follow a normal distribution

$$\mu = 2.73 \text{ log10 } OD_{50}\text{/dose } (\sigma = 0.14)$$

✓ Ability to detect batches with lower amount of antigen lower than the standard one:

- demonstrated through *linearity validation* protocol for linearity of the method

✓ confirmed by testing *sub-formulated batches:*

Experimental Vaccine	Mean ELISA titre (log_{10} OD_{50}/dose)	NIH Potency result (IU/dose)
Standard dose	2.76	(1)
½ dose	2.40	49.94
1/10 dose	1.69	6.96

(1) Not tested for ethical reason

95% within the range [μ-2σ ≤ X ≤ μ+2σ] => 97.5% *Release limit of acceptance ≥ 2.45 log10 OD50/dose*

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Establishment of limit of acceptance at end-of-shelf-life (…)

- ✓ State of the art *stability protocol on 3 batches* of RABISIN, including ELISA testing from batch release (T0) till at least three months over the approved shelf life (T39):

  Modelisation => *0.36 log10 OD50/dose* of average loss after 39m of storage

- ✓ Additional analysis of 7 batches of RABISIN at various advanced shelf-life (from 19m to 37m):

  Modelisation => *0.34 log10 OD50/dose* of average loss after 39m of storage

End-of-shelf-life limit of acceptance ≥ 2.09 log10 OD50/dose

(considering the release specification (2.45) and the average loss in titer (0.36))

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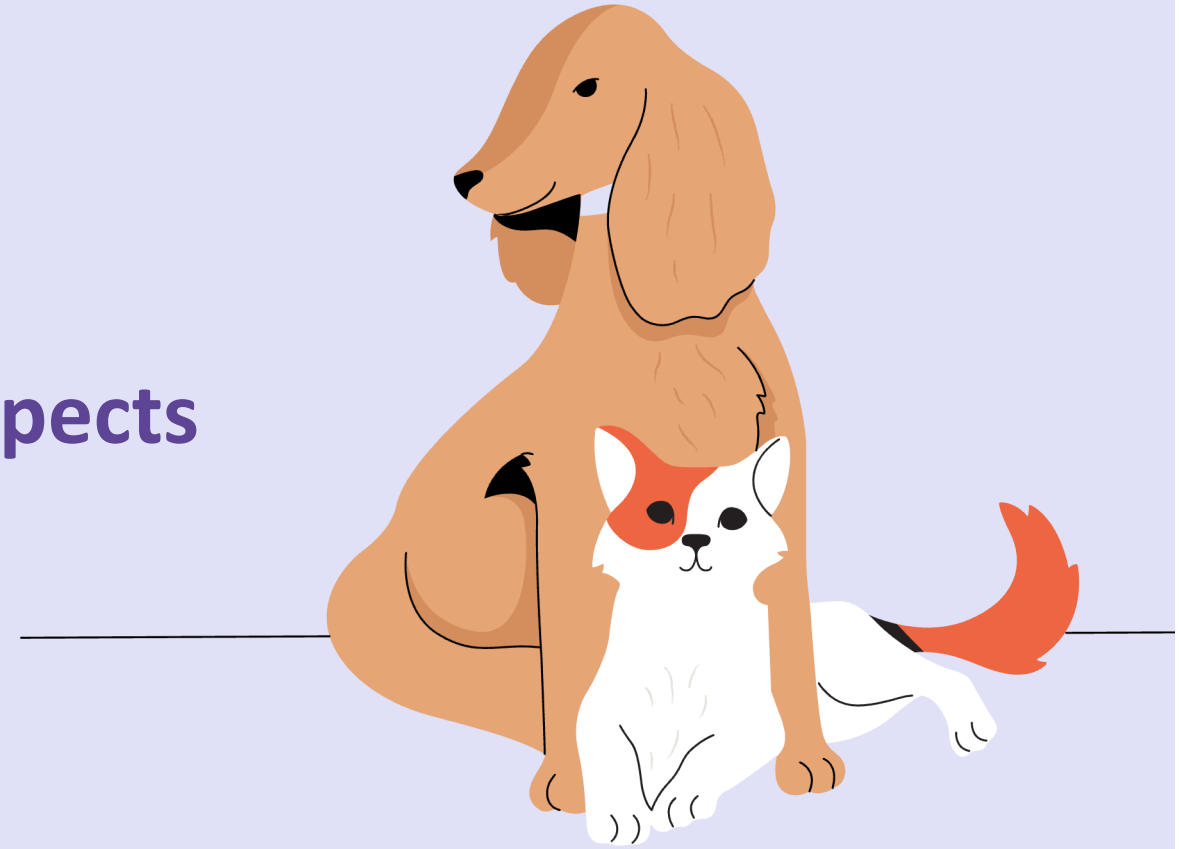
Regulatory Procedure aspects



RABISIN is registered worldwide through national procedures

✓ *In Europe*, the change was managed through 2 waves of variations using a Worksharing procedure with ANSES (France) as Reference Authorities.

- 2017: change in potency control test method for batch release (with release limit of acceptance),

- 2019, setting of an end-of-shelf-life limit of acetpance and consequential adaptation of the expression of the vaccine composition (SPC & leaflet)

✓ *Outside Europe*

✓ Variation *rejected or even not submitted* because of *NIH obligation*:

ARGENTINA, BRAZIL, CHINA, ECUADOR, INDIA, NICARAGUA, PERU, RUSSIA, THAILAND

✓ Variation *ongoing or approved anywhere-else*

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Thank you

AFSA/HealforAnimals webinars

Transition to non-animal based veterinary vaccine batch release testing. Policy and regulations theoretical aspects and case studies

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Life forward

In vitro potency test for Nobivac Rabies & RL

Development and licensing to replace an in vivo rabies potency test

Peter PLM Pepels, Ph.D.

- AFSA HA Webinar October 8, 2024

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Background information on product and in vivo test

- Nobivac Rabies since more than 3 decades licensed. Formulated on fixed amount of rabies Glycoprotein determined by ELISA.
- Potency of ≥ 2 IU/ml.
- Mouse challenge test replaced around 2015 by mouse VN-Ab test developed by PEI (Kraemer et al. 2010). Number of mice reduced from 168 to 16 per test / batch.
- Serology test still uses animals, live rabies virus in lab, long, biological test variability
- During IVP development PEI OMCL rabies group was consulted

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Challenges in development and for harmonisation IVP for vet. rabies vaccines

- Uncoupling G-protein from adjuvant is adjuvant specific (different between $AlOH_3$ and $AlPO_4$)
- Vet. rabies vaccines use different rabies strains: Ab ELISA panel used by other company did not work for Nobivac Rabies
- Ab for ELISA ideally only binds trimeric G-protein (and binds BRP standard well)

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IVP development based on G-protein recovery in vaccine

- Development strategy according Ph. Eur. 5.2.14. in four validation steps

i. Pretreatment method to release G-protein from adjuvant : high recovery

ii. Ph.Eur. 5.2.14: (i) quantifies antigen, (ii) detects antigen integrity, (iii) targets epitope relevant for protection

iii. AlphaLISA validation according VICH GL 1 & 2

iv. Release specifications setting: - consistency in historical challenge batch potency data &
- tolerance intervals of the IVP

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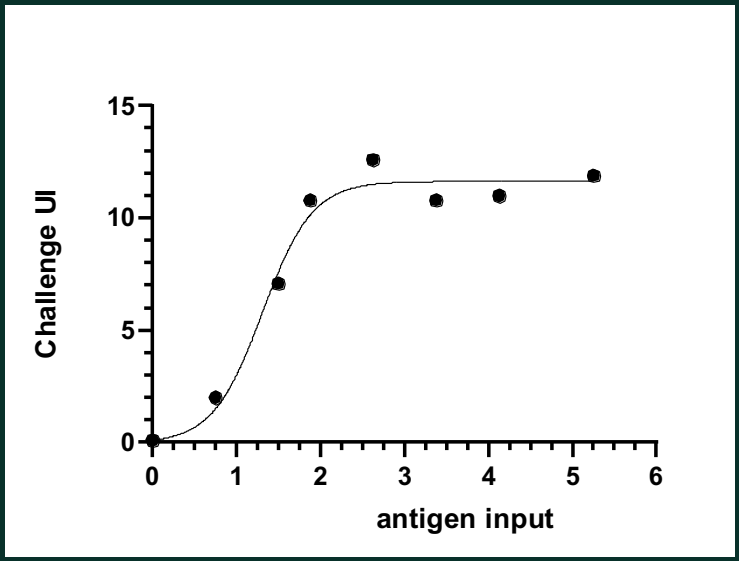
G-protein amount correlates with protection in mouse challenge test



- **G-protein/Ag vaccine input correlates with potency** (dose response for Nobivac Rabies)
- Well characterized **MoAb** only detects **native trimeric G-protein** = **major immunogen for VN-Ab formation**,
- MoAb does not bind denatured di or mono meric soluble glycoproteins that are poorly immunogenic.

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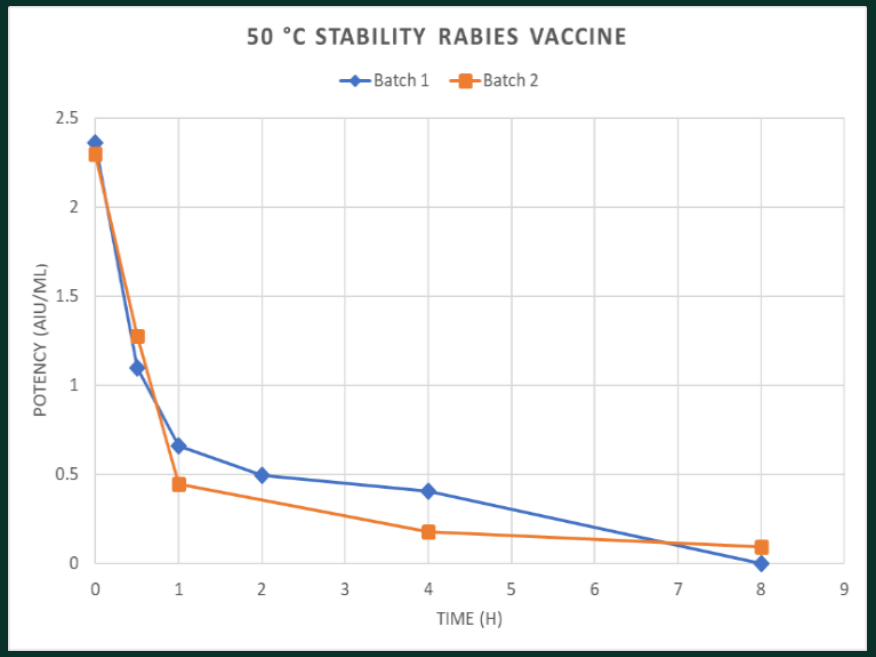
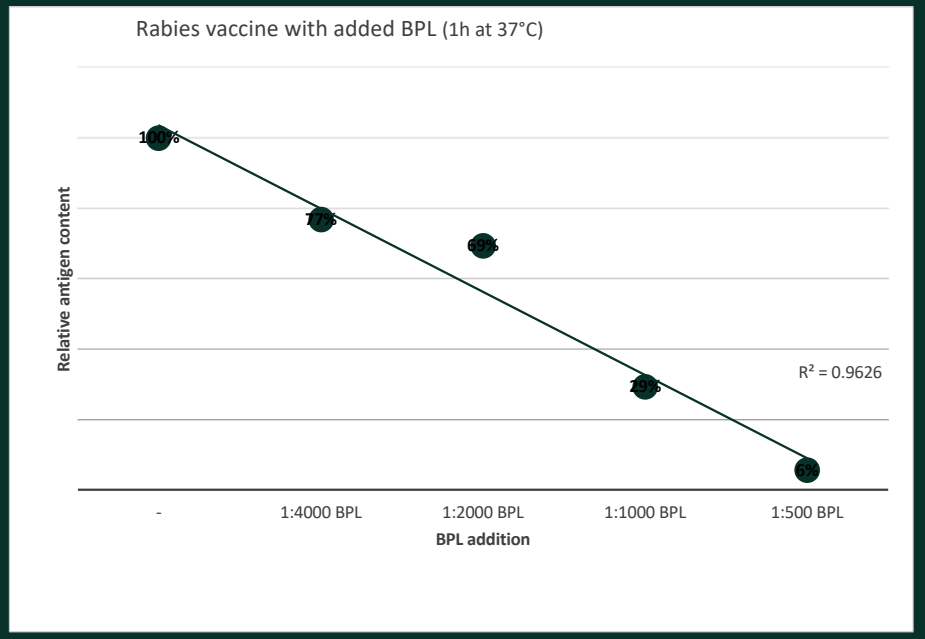
(ii) Ag integrity loss induced by heat or chemical treatment is well detected (discriminative power to detect sub-potent batches)

- **Heat treatment 50°C**



Chemical inactivation : Beta-propiolactone (BPL)



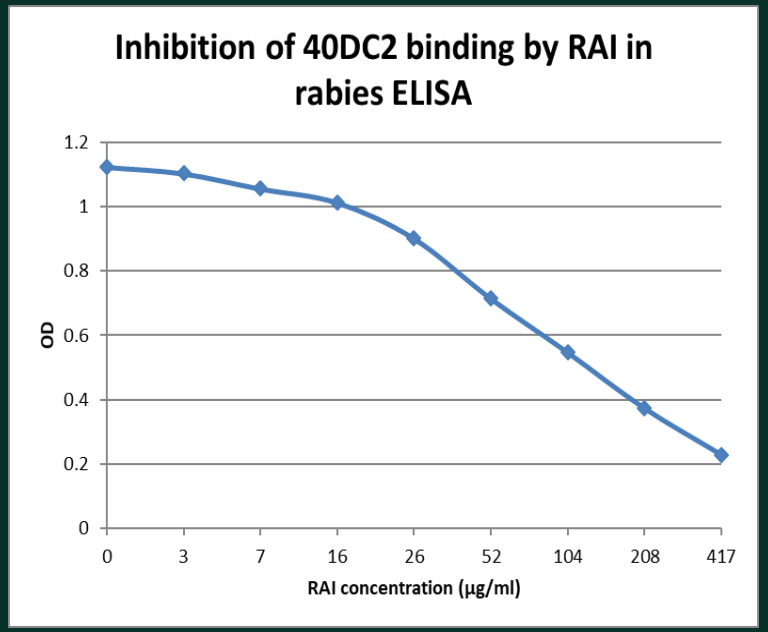
IVP MoAb targets epitope relevant for protection

- - MoAb used neutralises CVS in the RFFIT test
- MoAb competes with RAI binding in rabies ELISA
- RAI is the WHO 2nd International standard anti-rabies IgG

RAI			
Dilution	U/ml	+ wells	- wells
1:5	0.2	0	6
1:10	0.1	0	6
1:20	0.05	2	4
1:40	0.025	5	1
1:80	0.013	6	0
1:160	0.006	6	0
1:320	0.003	6	0
1:1600	0.001	6	0

40DC2			
Dilution	Concentration (µg/ml)	+ wells	- wells
1:5	242	0	6
1:10	121	0	6
1:20	61	0	6
1:40	30	0	6
1:80	15	0	6
1:160	7.6	0	6
1:320	3.8	0	6
1:1600	0.8	0	6

CVS Virus titration		
Dilution	+ wells	- wells
10^0	6	0
10^1	6	0
10^2	1	5
10^3	0	6
10^4	0	6
Titer: 1.67 (10log TCID50/50 µl)		



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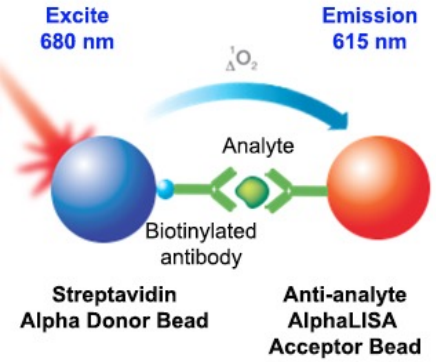
AlphaLISA Validation & Release specification setting

- **(iii) AlphaLISA passed validation requirements for :**
- G-protein concentration proportional to light emitted. Sample quantified against reference vaccine.
- Sample quantified against reference vaccine. Rapid, highly sensitive, no-wash alternative
- VICH validation passed: Linearity, Accuracy, Precision, Specificty & Robustness



- **(iv) Release specs were based on :**

1. 10 years historical challenge potency data analysis: high potency
2. AlphaLisa tolerance interval setting based on batches analyzed by Challenge and by AlphaLISA test

- **Workshare variation approval EU 2021 & outside EU from 2021 onwards**
- **IVP test succesfully transfered to PEI OMCL for official control authority batch release for Nobivac rabies and Nobivac Rabies Lepto**

Countries implemented / approved the IVP
(status September 2024)

Europe	Africa	Latin America	Asia	M. East	Australia / N Zealand
29 EU–member states countries & 7 Non-EU member state countries	7	16	12	6	2 (import permit)

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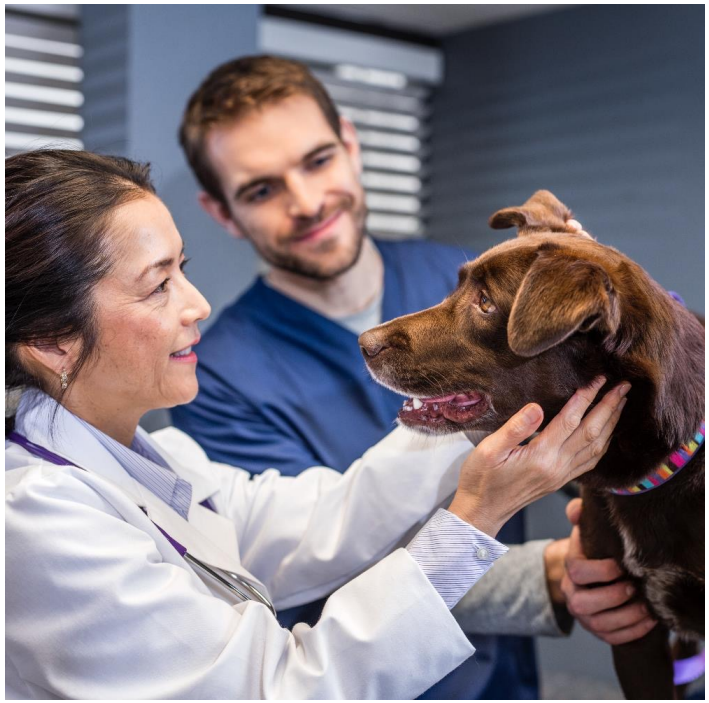
Thanks to the 3R group MSD:

Frits Hulshof, Imke Kross, Ester Piek

Vac2vac



Thank you

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Replacement of the current *in vivo* release potency/identity test on finished product for the rabies component by an alternative *in vitro* ELISA

October 2024

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Procedure EMEA/V/C/WS2184:

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

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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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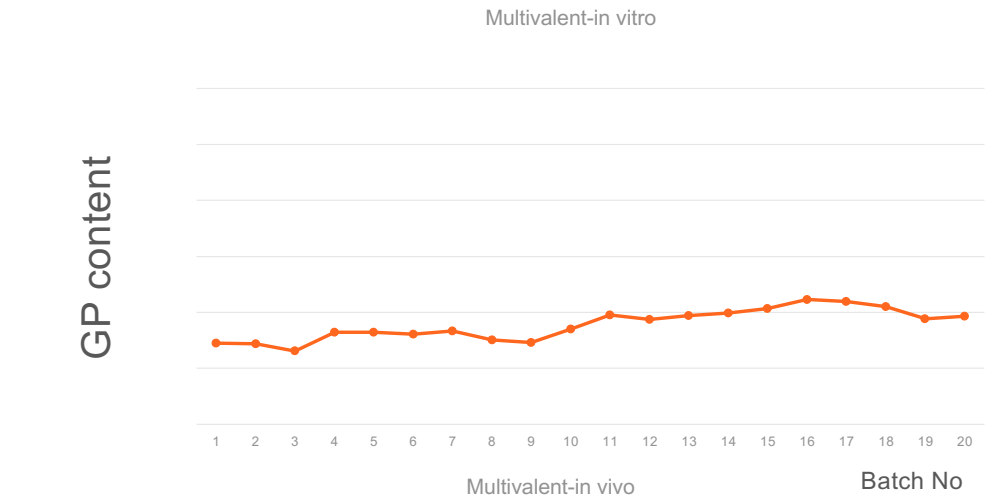
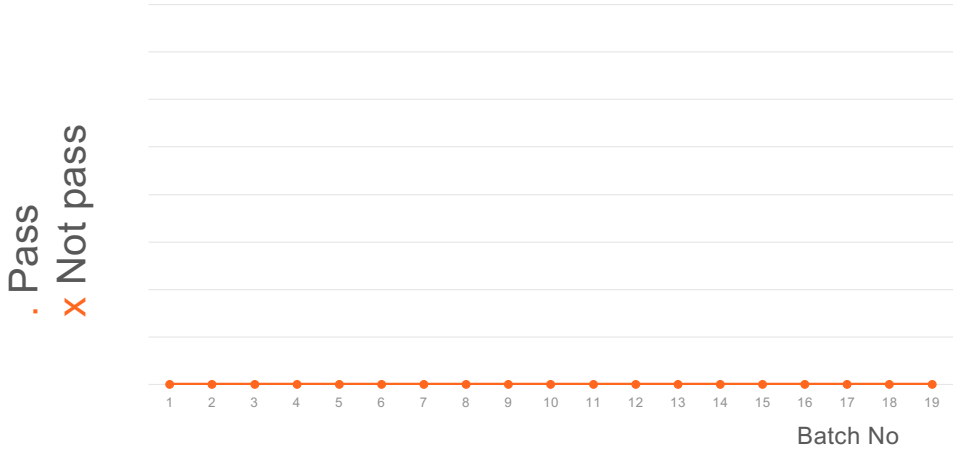
Zoetis's approach for response:

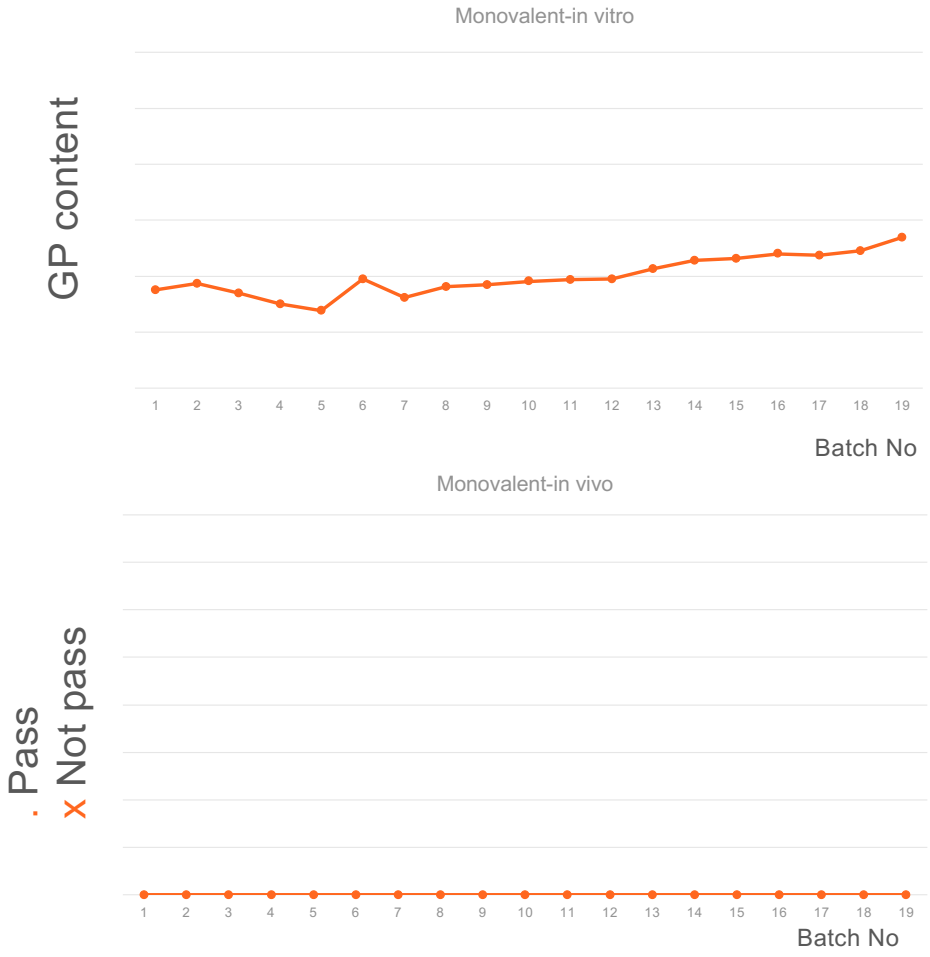
Consistency data





Zoetis's approach for response:

Consistency data



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

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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 2.77 IU/ml	Pass	Pass 5.20 IU/ml
#2	Pass	Pass	Not Pass	Not Pass	Pass	Pass 6.09 IU/ml	Pass	Pass 8.70 IU/ml
#3	Pass	Pass	Not Pass	Not Pass	Not Pass	Not Pass 1.23 IU/ml	Not Pass	Not Pass 3.10 IU/ml

ELISA Vs Serology

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

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MAIN QUESTIONS

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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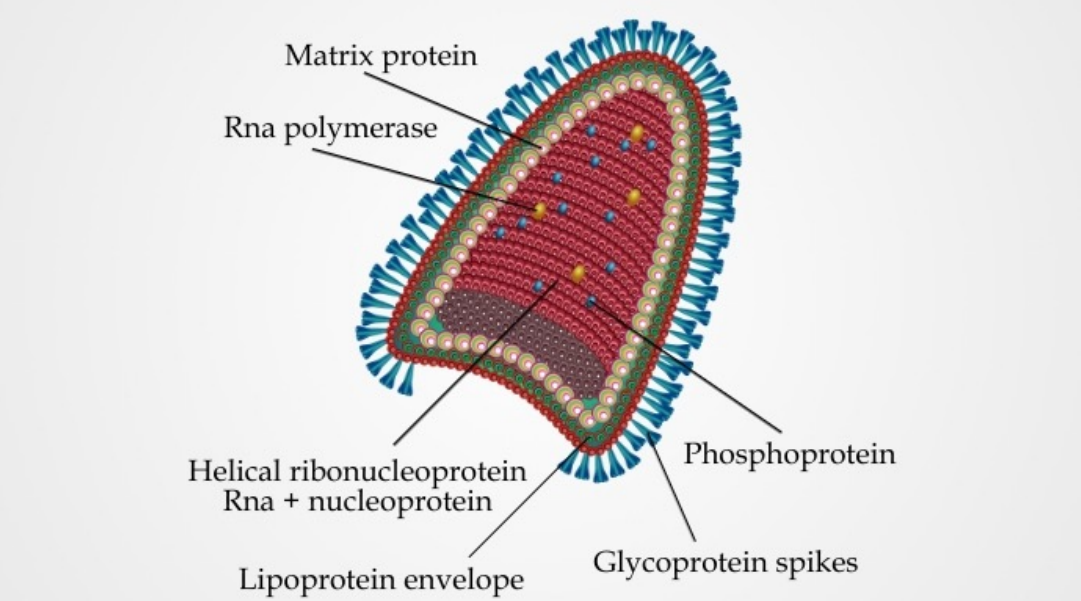
LITERATURE: STRUCTURE OF THE RABIES VIRUS

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

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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.**

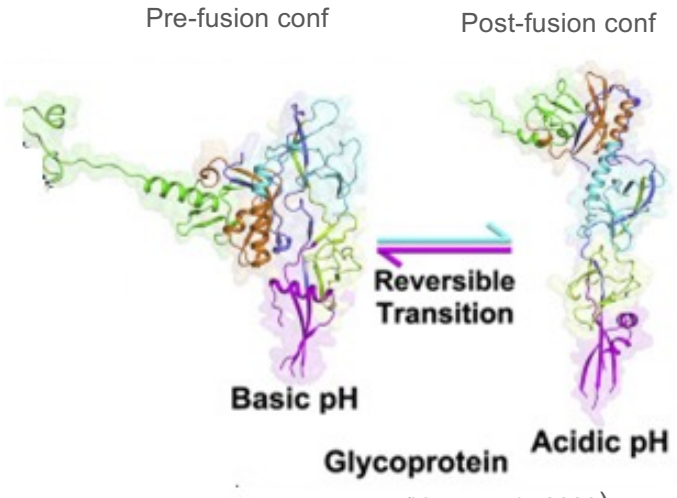
On the viral surface, RABV-G is structurally heterogeneous and **only a portion is recognizably trimeric.**

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)

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DEMONSTRATION OF THE SUITABILITY OF THE ASSAY: RELEVANT CONFORMATION DETECTION

Zoetis's approach for response:

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

Sample preparation:

10 ml Inactivated Vaccine rabies bulk → dialysed to different pH buffers to 12.5 ml:

- **50mM TRIS, pH=6,8, 150mM NaCl**
- **50mM Acetate, pH=4,8, 150mM NaCl**
- **50mM Acetate, pH=4,3, 150mM NaCl**
- **50mM Citrate, pH=3,8, 150mM NaCl**
- **50mM Citrate, pH=3,3, 150mM NaCl**

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

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Zoetis's approach for response:

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 less the GP detected by the ELISA

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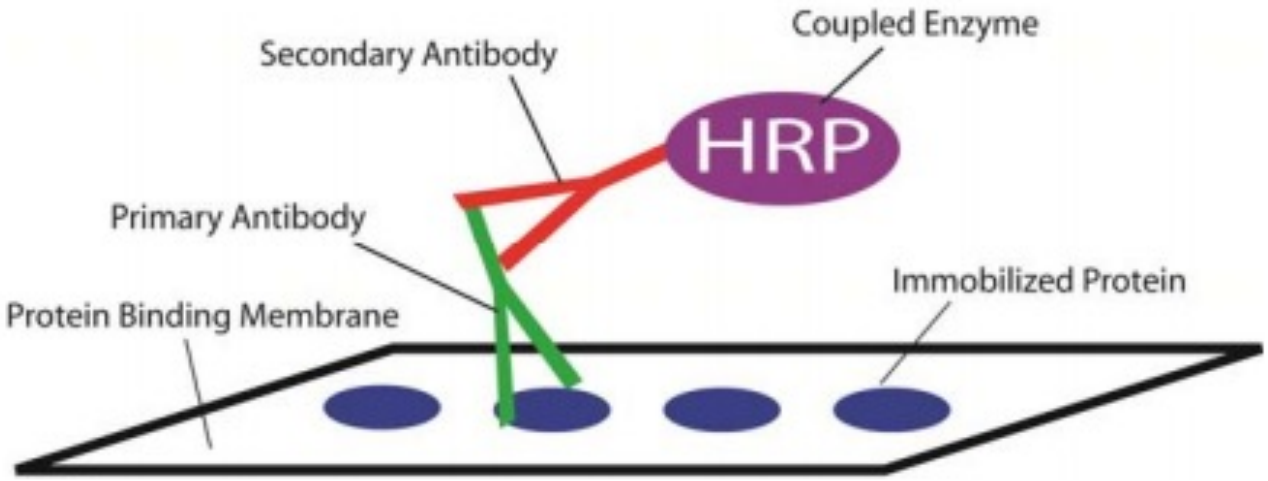
Zoetis's approach for response:

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

Dot Blot



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Zoetis's approach for response:

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

Manufacturing partner also has approvals in Ukraine, Turkey and Bosnia

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Thank you!

Conclusion

In all examples you see 3 key aspects that were critical to succuss

- Demonstrating the suitability of the method to detect and immunologically relevant antigen
- Establishing discriminatory power – ensuring the method could detect both sub-potent and sub-standard batch
- Using the consistency approach to establish relevant release and end of shelf-life specifications

Note – there was no correlation established between the *in vivo* and *in vitro* methods, but they were in some cases shown to be consistent with regards pass/fail results

Key Messages

- One single assay method may not be suitable for all products
  - The BI method was tested by all manufacturers during VAC2VAC and did not work or all
  - Using the same approach in all cases increases confidence in both the approach and outcome
- 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
- All 3 methods have been accepted in countries globally
- 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 supply issues
    - Less no tests or test failures so less repeat testing
    - Time (more shelf-life on product)
- In the Rabies case a relevant epitope is well established and defined but this will not always be the case

Thank you!

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