Rabies Batch Release Testing

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First DCVMN 3Rs Experts Working Group Meeting

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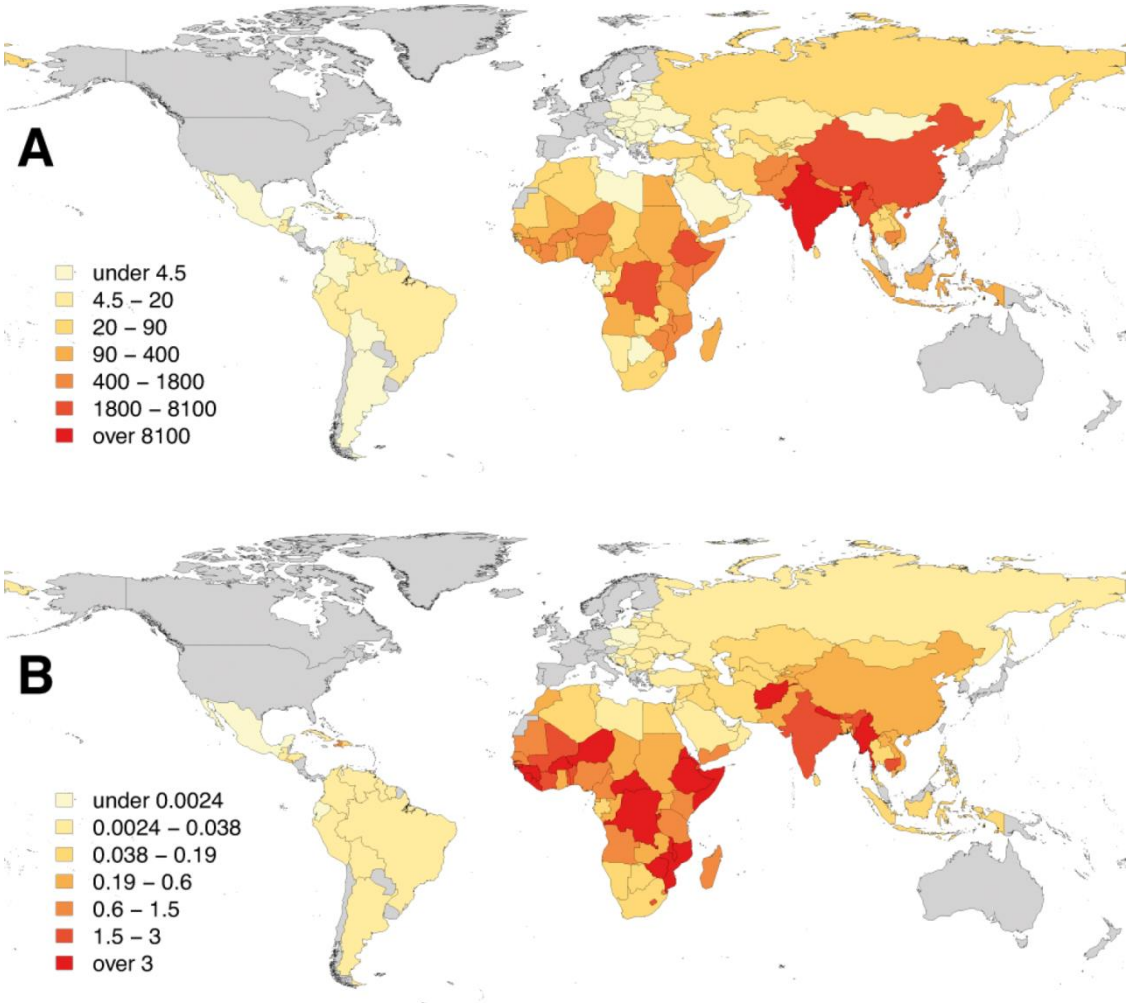
❑Overview

- Introduction
- Epidemiology and burden of disease
- Current Vaccines and Process
- 3R Strategy
- Development of ELISA method (G-Protein)
- Conclusion

❑ Introduction

- Rabies causes an estimated 59,000 mortalities every year
- That's approximately 1 death every 15 minutes
- Human Rabies is present in 150 countries and territories and on all continents, except for Antarctica
- 80% of cases occur in individuals living in rural populations- most of which are children
- Domestic dogs are the most common reservoir of the virus, with more than 95% of human deaths caused by dogs
- 95% of cases are reported in Asia and Africa

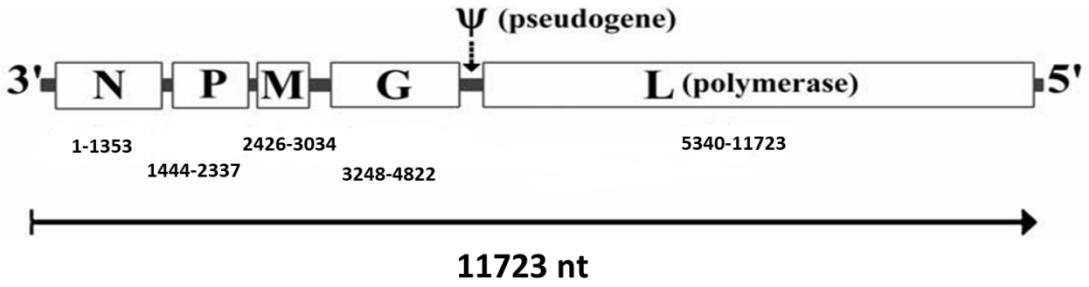
❑ Epidemiology and burden of disease

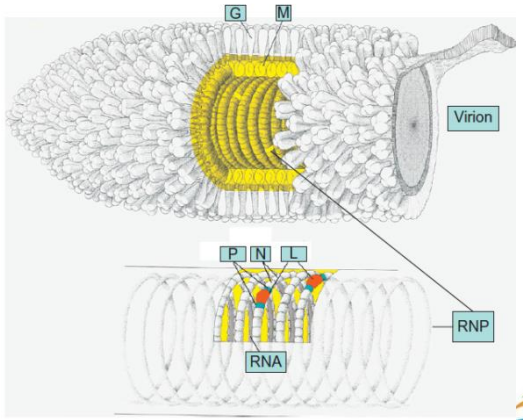


A. Human Deaths form Rabies; B. Death rates per capita (per 100 000 population); countries shaded in grey are free from canine Rabies
https://www.who.int/rabies/epidemiology/en/

❑ Rabies virus (RABV)

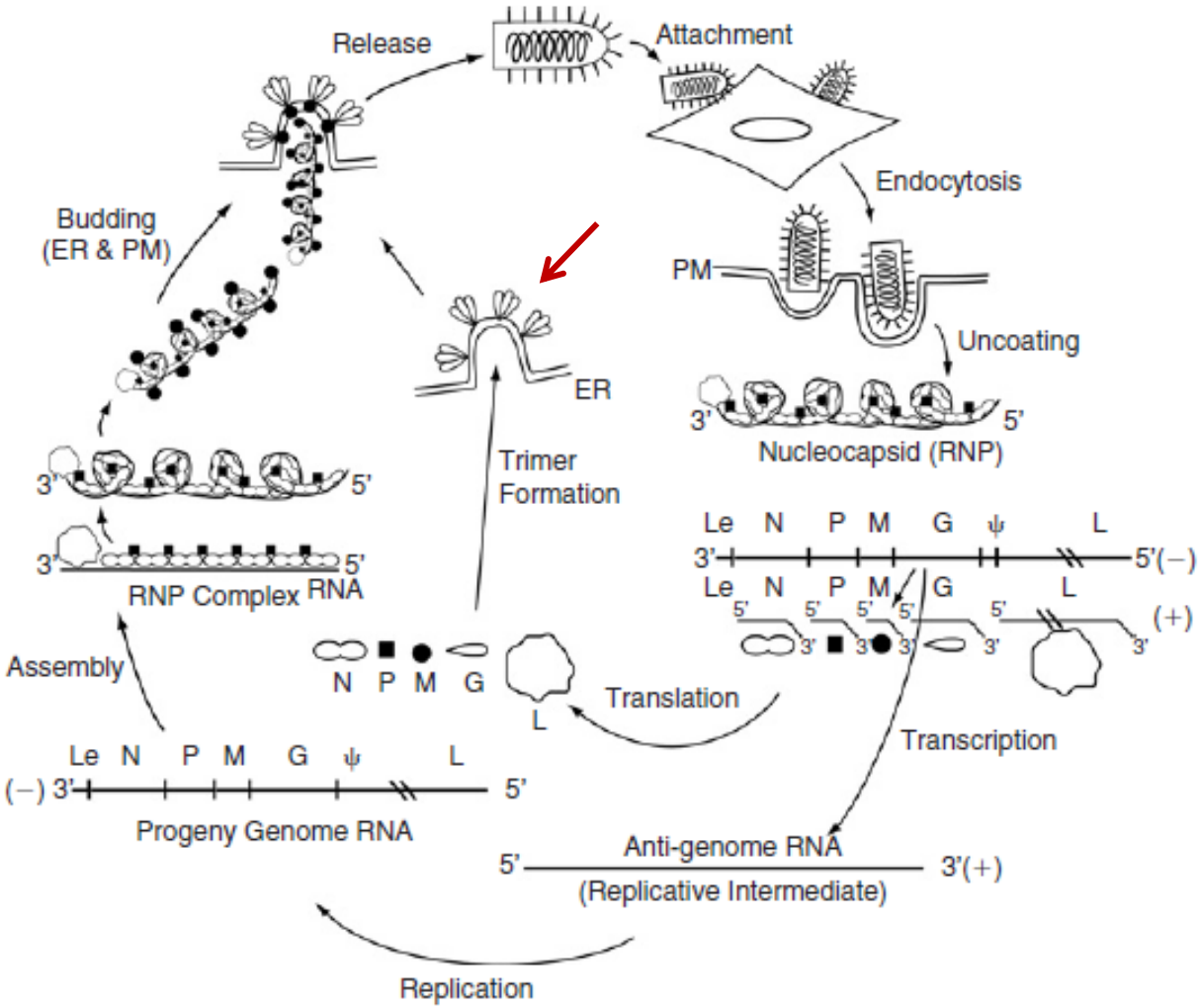
- Rabies virus (RABV) is the prototype virus of the genus *Lyssavirus* (from the Greek *lyssa* meaning 'rage') in the family *Rhabdoviridae* (from the Greek *rhabdos* meaning 'rod').
- RABV is a highly neurotropic virus in the infected mammalian (animal and human) host, invariably causing a fatal encephalomyelitis.
- Organization of the rabies virus genome has Nucleoprotein (N), Phosphoprotein (P), Matrix protein (M), Gycoprotein (G) and large RNA-polymerase protein (L) genes are separated by intergenic di- and penta-nucleotide sequences and the long pseudogene (Ψ) sequence and are flanked by the leader (Le) RNA and trailer (Tr) RNA sequences at the 3' and 5' ends.





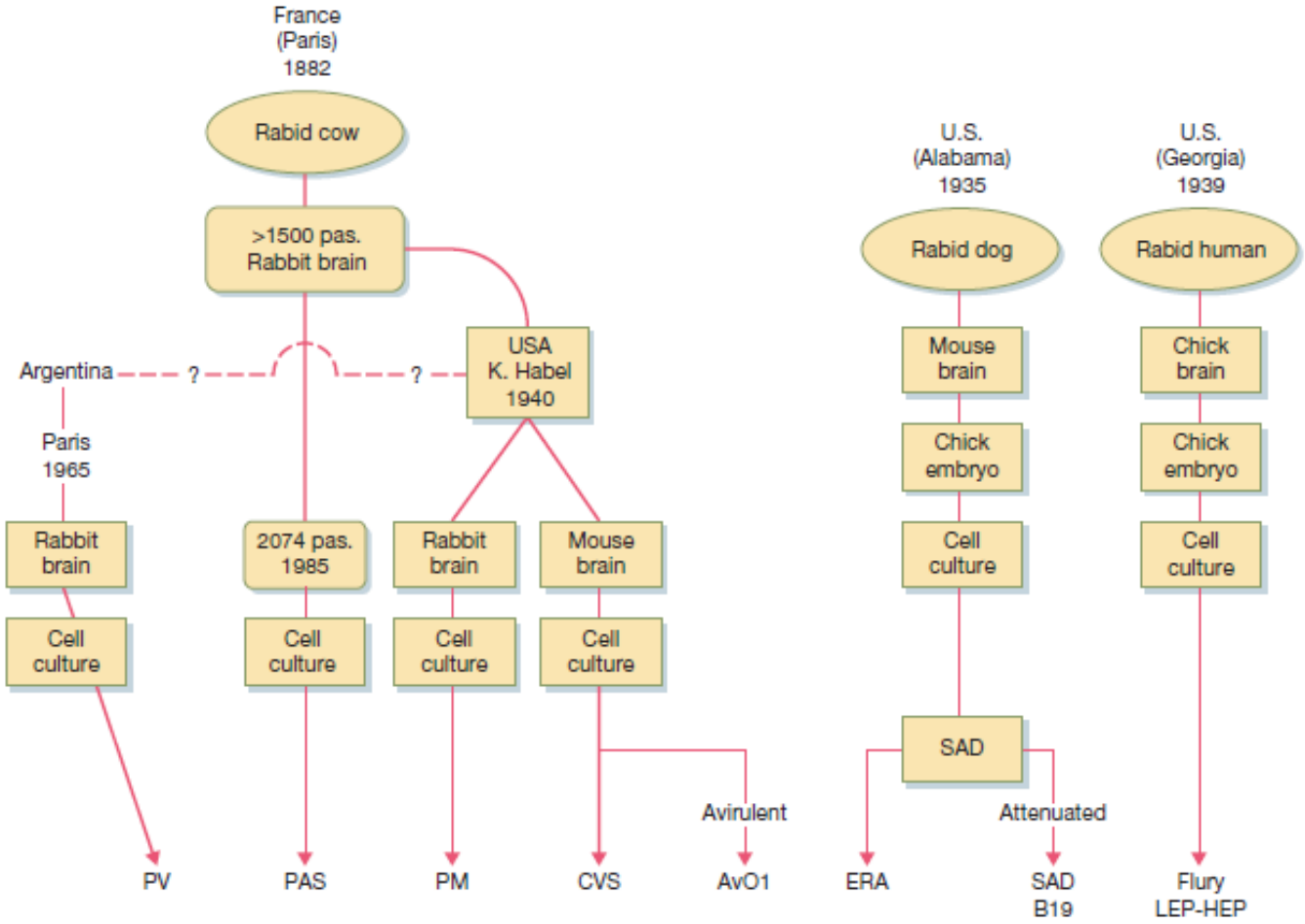
Review of Infectious Diseases, Wunner, et al., Supplement 4, S771–S784, 1988,

❑ Rabies Virus Life Cycle



Ref: Rabies Second Edition, Alan C. Jackson and William H. Wunner

❑ History of strains of rabies viruses used as vaccine seeds



PAS, Pasteur; PM, Pitman-Moore; PV, Pasteur virus; SAD, Street Alabama Dufferin

Ref. Plotkin's - Vaccines

❑ Brief Manufacturing Process (Zydus)

1. SPF Chicken Egg receipt & Incubation
2. Candling, Embryo collection and pooling
3. Trypsinization and Preparation of cell suspension
4. Rabies Virus Infection and Adsorption
5. Virus Harvest
6. Pooling and Filtration of Virus Harvest
7. Purification and Concentration of virus Harvest
8. Inactivation of Bulk
9. Final Bulk Vaccine preparation and blending
10. Filling and Lyophilisation
11. Finished Product

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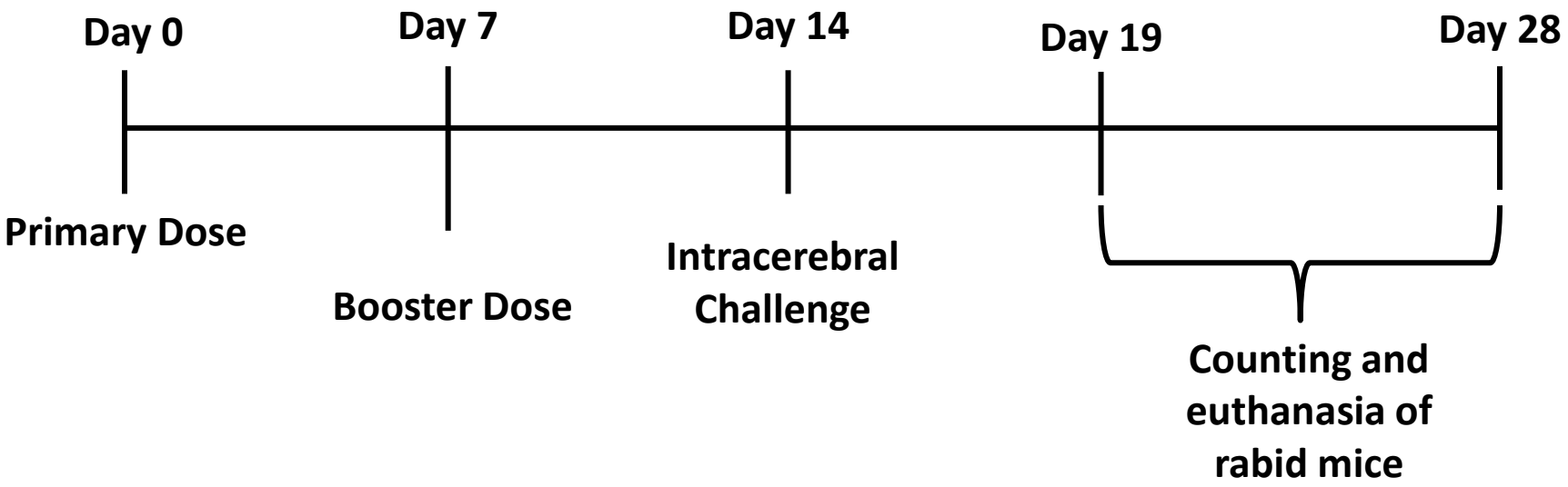
❑ Rabies NIH Potency assay (Finished Product)

Sample Dilution	
1/25	1/625
1/125	1/3125

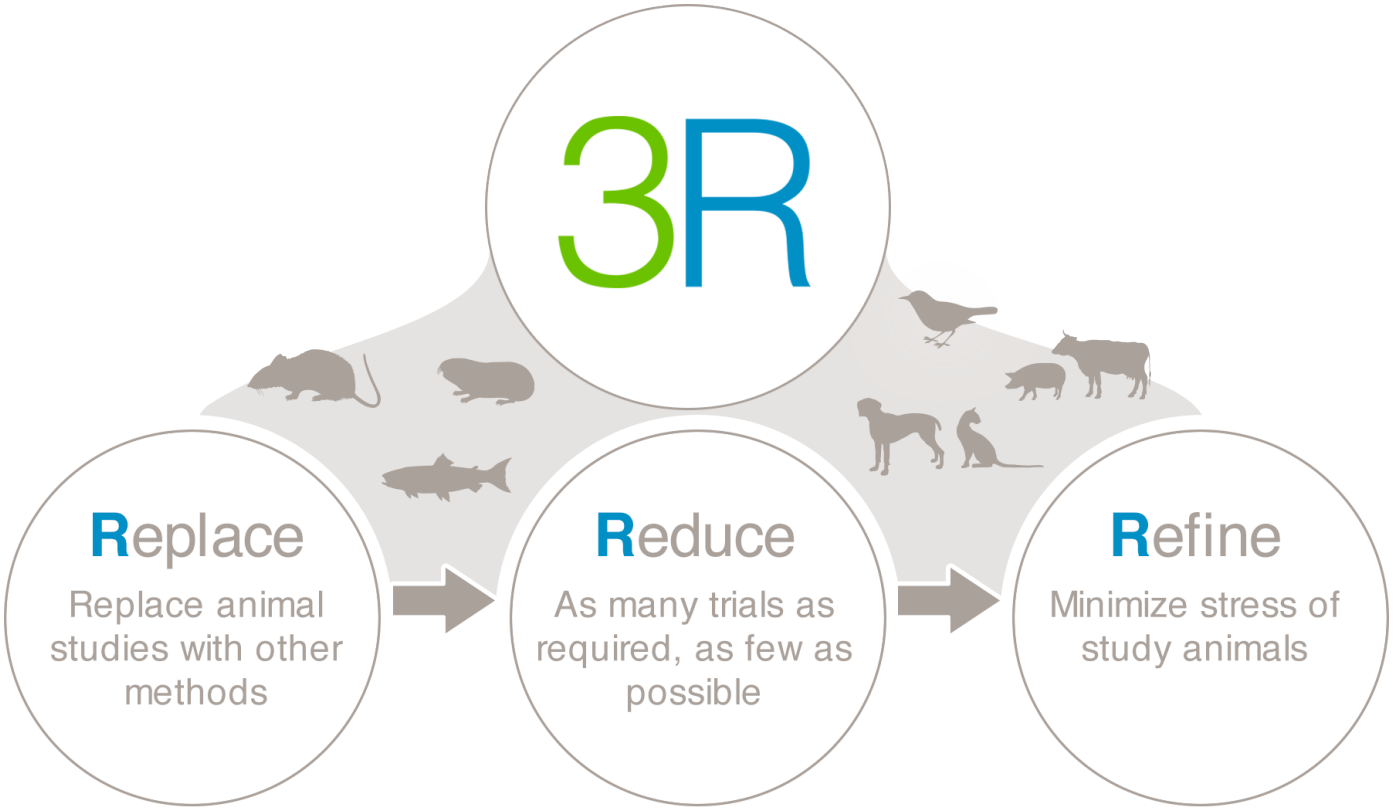


- Worldwide used release test, specification≥2.5 IU per single human dose
- Ph.Eur. 0216, WHO TRS 941

❑ Issues and challenges with the *in vivo* challenge (NIH) test

- Time consuming 28 days to complete the test
- Very high variability : 25-400%
- Hazardous- Safety issues- Need for BSL3 containment due to the use of live rabies virus
- Purity of the animal strain/breed
- Animal usage – Approx. 150 animals per test
- Availability of CVS (challenge virus strain)
- Regulators: in most of the Regulatory Guidelines, NIH is mandatory for final lot release

❑ Alternatives to Animal Experiments



W. M. S. Russell and R. L. Burch in 1959

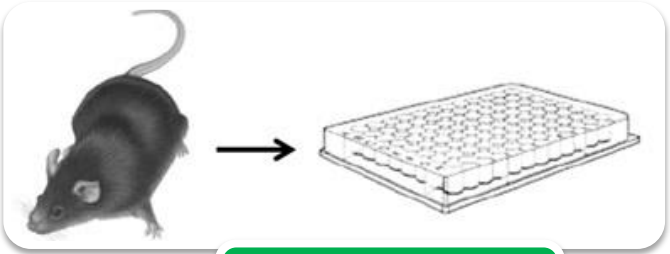
❑ Global Scenario on alternate strategy to NIH

- ❑ The *in-vitro* ELISA, as an alternative to the NIH test, is :
  - ✓ in accordance with the Ph. Eur. 3Rs strategy: replacement
  - ✓ already used by some manufacturers for blending and monitoring of the consistency of production
- ❑ EPAA (European partnership for alternative approaches in animal testing) has already started a study with industrial collaborative partners to replace Human Rabies potency test (Project Code BSP148) which is been very well supported by all vaccine manufacturers and WHO for Harmonization and make it a release test

❑ Immunogenicity Assay/Potency Assays

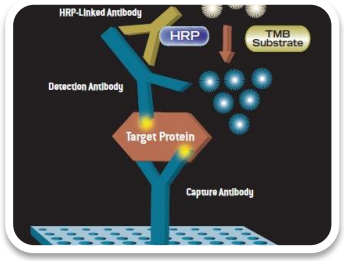
In-vivo

- In-vivo: Early 1900s Lethal Challenge in Animal models (Animal challenge study)
- High cost, time , labour, resources yet high variability.



In-vivo/ In-vitro

- In-Direct ELISA
- Neutralization Assay
- PRNT



In-vitro

- SRID (Influenza)
- ELISA- In-vitro Potency assay to check antigen using specific monoclonal antibody

❑ Development of in-vitro potency assay

- ❑ Zydus being one of the vaccine manufacturers of human rabies vaccine has developed an *in-vitro* potency assay based on G-Protein
- ❑ Validation of the method with the different batches are under progress

❑ Publications (1985-2019)

Title	Author	Year
Use of a monoclonal antibody for quantitation of rabies vaccine glycoprotein by enzyme immunoassay.	*Lafon M, et al.,*	J Biol Stand. 1985 Oct;13(4):295-301.
Standardization of an enzyme immunoassay for the in vitro potency assay of inactivated tissue culture rabies vaccines: determination of the rabies virus glycoprotein with polyclonal antisera.	*Thraenhart O, et al.,*	J Biol Stand. 1989 Oct;17(4):291-309.
In vitro rabies vaccine potency appraisal by ELISA: advantages of the immunocapture method with a neutralizing anti-glycoprotein monoclonal antibody.	*Perrin P, et al.,*	Biologicals. 1990 Oct;18(4):321-30.
A relevant in vitro ELISA test in alternative to the in vivo NIH test for human rabies vaccine batch release.	*Gibert R, et al.,*	Vaccine. 2013 Dec 5;31(50):6022-9.
G-protein based ELISA as a potency test for rabies vaccines.	*Chabaud-Riou M, et al.,*	Biologicals. 2017 Mar;46:124-129.
Replacement of in vivo human rabies vaccine potency testing by in vitro glycoprotein quantification using ELISA - Results of an international collaborative study.	*Morgeaux S, et al.,*	Vaccine. 2017 Feb 7;35(6):966-971.
Development of a relative potency test using ELISA for human rabies vaccines.	*Wang Z, et al.,*	Biologicals. 2018 Sep;55:59-62.
Potency test to discriminate between differentially over-inactivated rabies vaccines: Agreement between the NIH assay and a G-protein based ELISA.	*Toinon A, et al.,*	Biologicals. 2019 May 17.

❑ G-Protein ELISA

Biologicals 46 (2017) 124–129

Contents lists available at ScienceDirect

Biologicals

journal homepage: www.elsevier.com/locate/biologicals

G-protein based ELISA as a potency test for rabies vaccines

Martine Chabaud-Riou, Nadège Moreno, Fabien Guinchard, Marie Claire Nicolai, Elisabeth Niogret-Siohan, Nicolas Sève, Catherine Manin*, Françoise Guinet-Morlot, Patrice Riou

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ABSTRACT

The NIH test is currently used to assess the potency of rabies vaccine, a key criterion for vaccine release. This test is based on mice immunization followed by intracerebral viral challenge. As part of global efforts to reduce animal experimentation and in the framework of the development of Sanofi Pasteur next generation, highly-purified vaccine, produced without any material of human or animal origin, we developed an ELISA as an alternative to the NIH test. This ELISA is based on monoclonal antibodies recognizing specifically the native form of the viral G-protein, the major antigen that induces neutralizing antibody response to rabies virus. We show here that our ELISA is able to distinguish between potent and different types of sub-potent vaccine lots. Satisfactory agreement was observed between the ELISA and the NIH test in the determination of the vaccine titer and their capacity to discern conform from non-conform batches. Our ELISA meets the criteria for a stability-indicating assay and has been successfully used to develop the new generation of rabies vaccine candidates. After an EPAA international pre-collaborative study, this ELISA was selected as the assay of choice for the EDQM collaborative study aimed at replacing the rabies vaccine NIH *in vivo* potency test.

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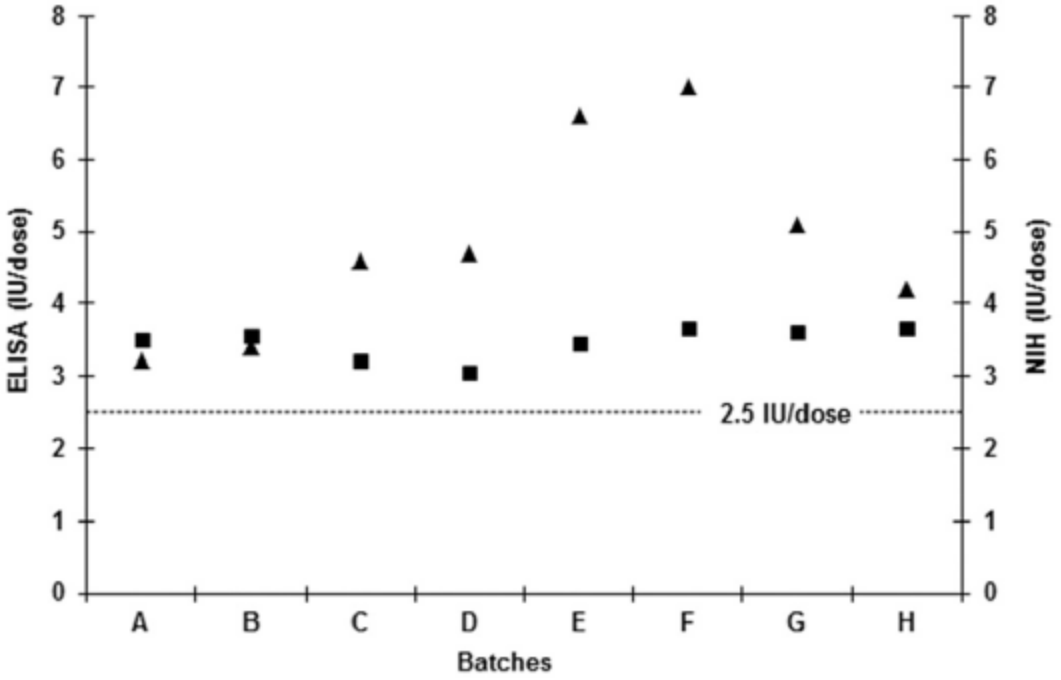
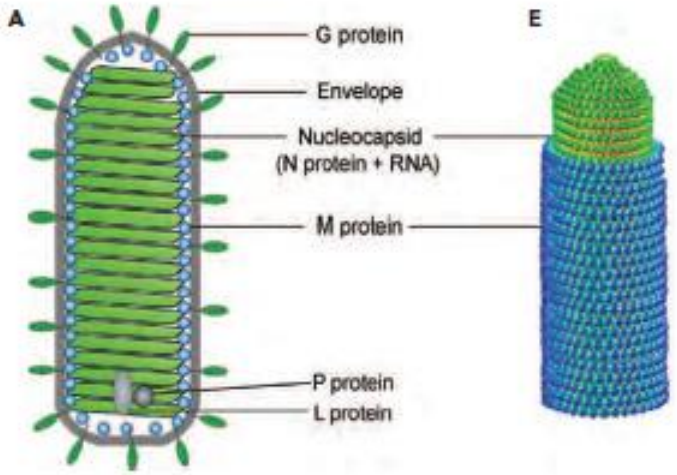


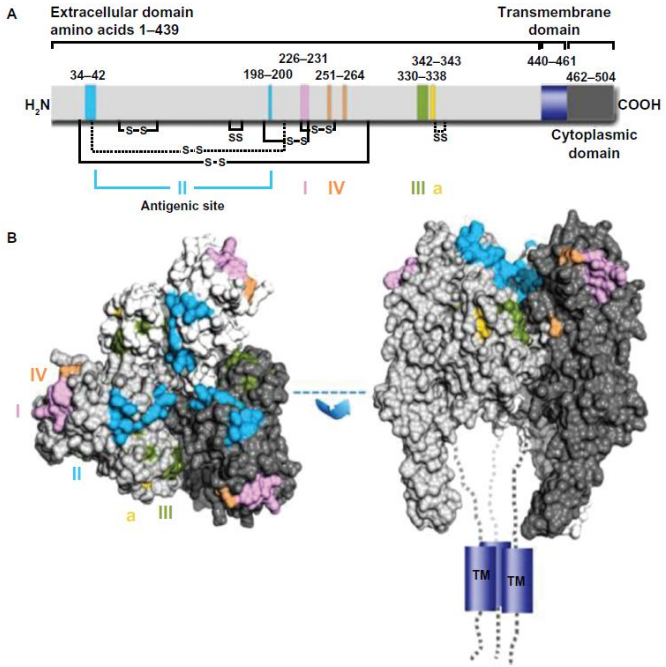
Fig. 2. Comparative analysis of different lots of rabies vaccine at the Filled Product step. The horizontal bar corresponds to the threshold for conformity. Triangle and right-hand y axis: NIH test; Square and left-hand y-axis: ELISA assay.

Riou et al., 2017

❑ G Protein



Fields virology/editors-in-chief, David M. Knipe, Peter M. Howley. – 6th ed.



Nagarajan et al., 2014

- G Protein, which is a trimer of approximately 67kDa, is the major antigen responsible for inducing production of VNAs and for conferring immunity against lethal infection with rabies virus

❑ *In-vitro* Potency assay

- Serum Antibody Assay

  G-Protein ELISA (In-direct ELSIA or c-ELISA)

Challenge can be avoided

- Antigen ELISA
  - ➢ Sandwich ELISA
    - ✓ Polyclonal Sera
    - ✓ Monoclonal ELISA
    - ✓ Polyclonal and Monoclonal ELISA

In-vitro Potency assay

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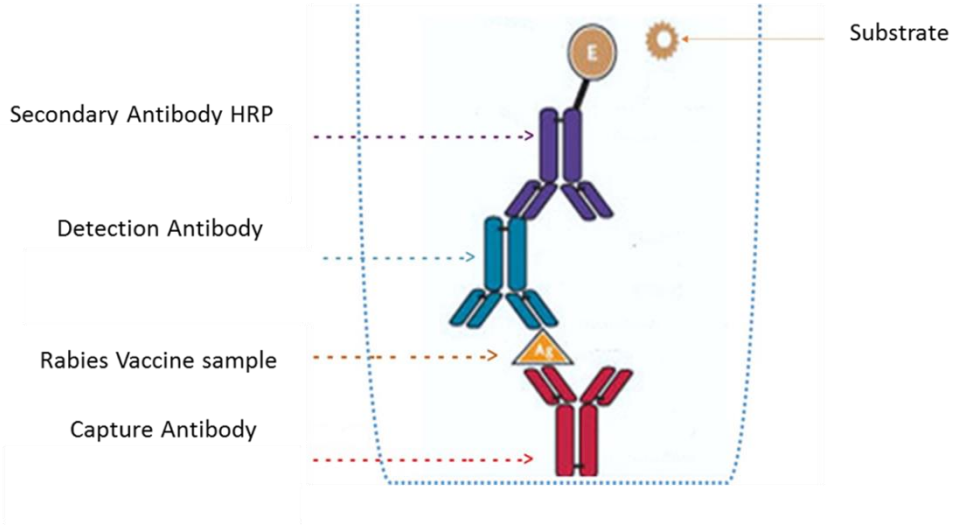
❑ In-vitro Assay Platform

Sandwich ELISA



Critical Reagent:

1. Capture antibody
2. Detection antibody
3. Reference standard

❑ Identification of monoclonal antibody

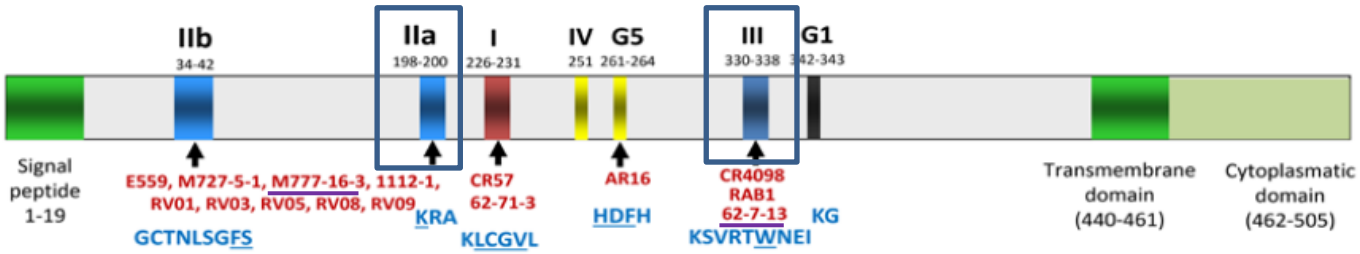


Figure 1: Schematic representation of the RV glycoprotein. Major antigenic sites and their amino acid positions are shown above the bar. Arrows indicate MAb epitopes within antigenic sites. The MAbs addressed in this study are shown in red. Consensus amino acid sequences of the antigenic sites are shown in blue. Underlined residues are invariable.

1.M777-16-3 **(IgG1)** binds to **Site IIa**

2.62-71-3 **(IgG2b)** binds to **Site III**

❑ Technical Information of the Monoclonal antibody

OPEN ACCESS Freely available online

PLoS NEGLECTED TROPICAL DISEASES

Development of a Mouse Monoclonal Antibody Cocktail for Post-exposure Rabies Prophylaxis in Humans

Thomas Müller[1], Bernhard Dietzschold[2], Hildegund Ertl[3], Anthony R. Fooks[4], Conrad Freuling[1], Christine Fehlner-Gardiner[5], Jeannette Kliemt[1], Francois X. Meslin[6], Charles E. Rupprecht[7], Noël Tordo[8], Alexander I. Wanderler[5], Marie Paule Kieny[9]*

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❑ Technical Information of the Monoclonal antibody

Table 1. Available technical information for candidate MoMAbs.

History of hybridomas	E559.9.14	1112-1	62-7-13	M727-5-1	M777-16-3
Mouse strain providing B-cells	BALB/c mice	BALB/c mice	BALB/c mice	BALB/c mice	BALB/c mice
Antigen	ERA G protein	ERA G protein	whole ERA	whole ERA, #167–169	whole ERA, #167–169
Fusion partner (Year of fusion)	P3-X63Ag8 (1979)	653 (1985)	Sp2/0–Ag14 myeloma (1983)	Sp2/0–Ag14 myeloma (1994)	Sp2/0–Ag14 myeloma (1994)
Reference	[35]	[50]	no	no	no
Number of cloning steps	4	Not known	3	4	4
Purity/homogeneity of cell line	Not known	Not known	Sub-cloned 2x, single IgG peak	isotype as pure IgG 2a	isotype as pure IgG 1
Origin of FCS used	New Zealand	USA	USA (GIBCO)	USA (Sigma), Canada (Wisent)	USA (Sigma), Canada (Wisent)
Absence of adventitious agents	Mycoplasma free	n.d.	Per WHO screening request	n.d.	n.d.
Culture conditions					
Medium	Iscove's DMEM 1	DMEM (modified)	Iscove's DMEM 2	HY-HT (10% FCS)	HY-HT (10% FCS)
Cell concentration	10^4–10^6	10^4–10^6	2×10^5	6×10^4–3×10^5	7×10^4–3×10^5
Serum-free culture medium	CD HM or PFHM II protein-free	Not tested	tested but no specification	Ultradoma-PF	Ultradoma-PF
Type of immunoglobulin					
IgG subtytpe	IgG 1 (ELISA)	IgG 1 (ELISA)	IgG 2b (ELISA)	IgG2a (FCA)	IgG 1 (FCA)
Heavy/light chains cDNAs	Yes	Yes	no	no	no
Antigenic site recognized on G	II	II c	III	II	II
Method for determining epitope	sequencing	sequencing	cross-neutralisation	cross-neutralisation	cross-neutralisation
Escape mutants					
derivation	SAD B19	CVS-11	not available	not available	ERA
aa substitutions in G	aa 57 (Leu to Arg)	aa 53 (Gly to Glu)			aa 198 (Lys to Glu)
	aa 217 (Lys to Glu)				aa 286 (Ala to Thr)
Production yield					
Yield in IU/ml (crude hybridoma)	62.5	3	30–60	22–32	11–32

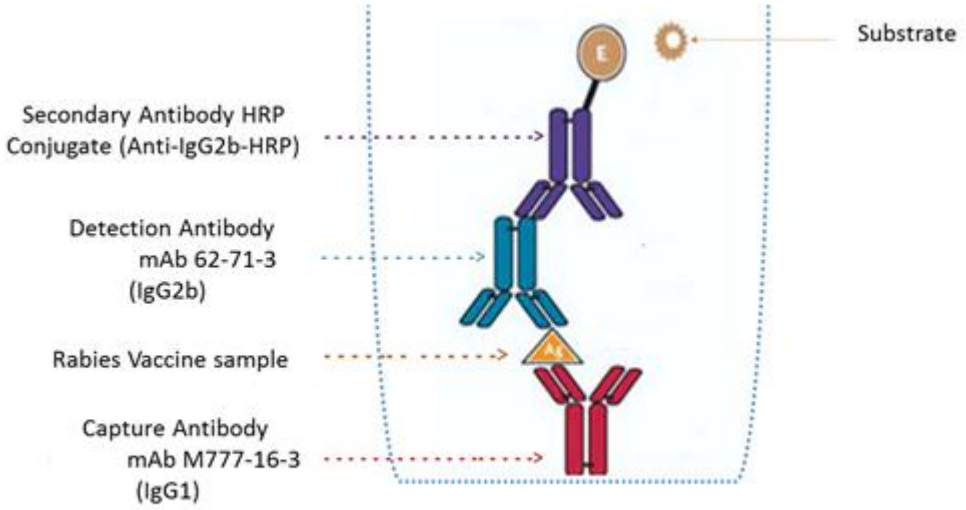
Legend: aa–amino acid, CVS 11–Challenge virus standard 11, DMEM–Dulbeccos' minimum essential medium, ELISA–enzyme linked immunosorbent assay, ERA–Evelyn Rokitniki Abelseth SAD derived RABV strain, FCA–Flouricon-CA Assay, HB–hybridization medium, SAD–Street Alabama Dufferin strain of RABV. Media specification: Iscove's DMEM 1 = Iscove's modified DMEM + HAM F12 (1:1) + 10% FCS; Iscove's DMEM 2 = Iscove's modified DMEM + ITS + antibiotics/antimycotics + L-glutamine + 5% FCS.
doi:10.1371/journal.pntd.0000542.t001

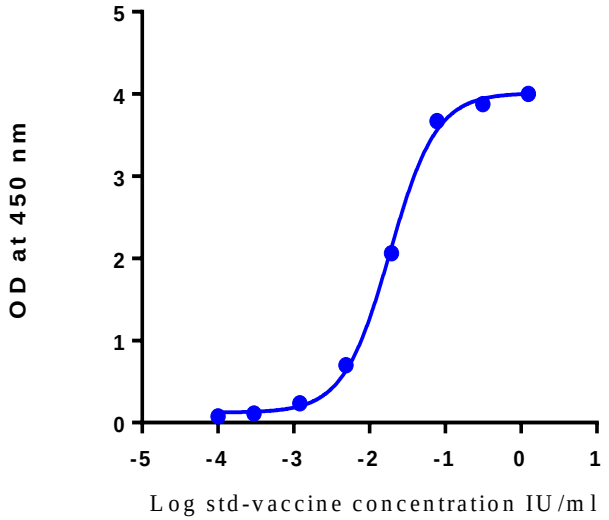
❑Development of G-Protein SW-ELISA method for Rabies vaccine testing

➢In-house highly characterized mAbs binding to the G protein of Rabies antigen

1.M777-16-3 **(IgG1)** binds to **Site II a (Capture Antibody)**

2.62-71-3 **(IgG2b)** binds to **Site III (Detection Antibody)**





❑ Sandwich ELISA Procedure

Step 1: Coating of Rabies capture antibody (Site II) Incubate the ELISA plate at 2-8°C for overnight

Day 1

↓

Step 2: 200µL of blocking solution to each well of the ELISA plate and incubate for 1 hrs ± 5 min at 37 ºC± 2° C

↓

Step 3: Add standard and sample in different dilutions

↓

Step 4: Add Detection antibody (Site III)

↓

Step 5: Add anti antibody HRP conjugated

Day 2

↓

Step 6: Substrate , stop reaction and read plate at 450nm

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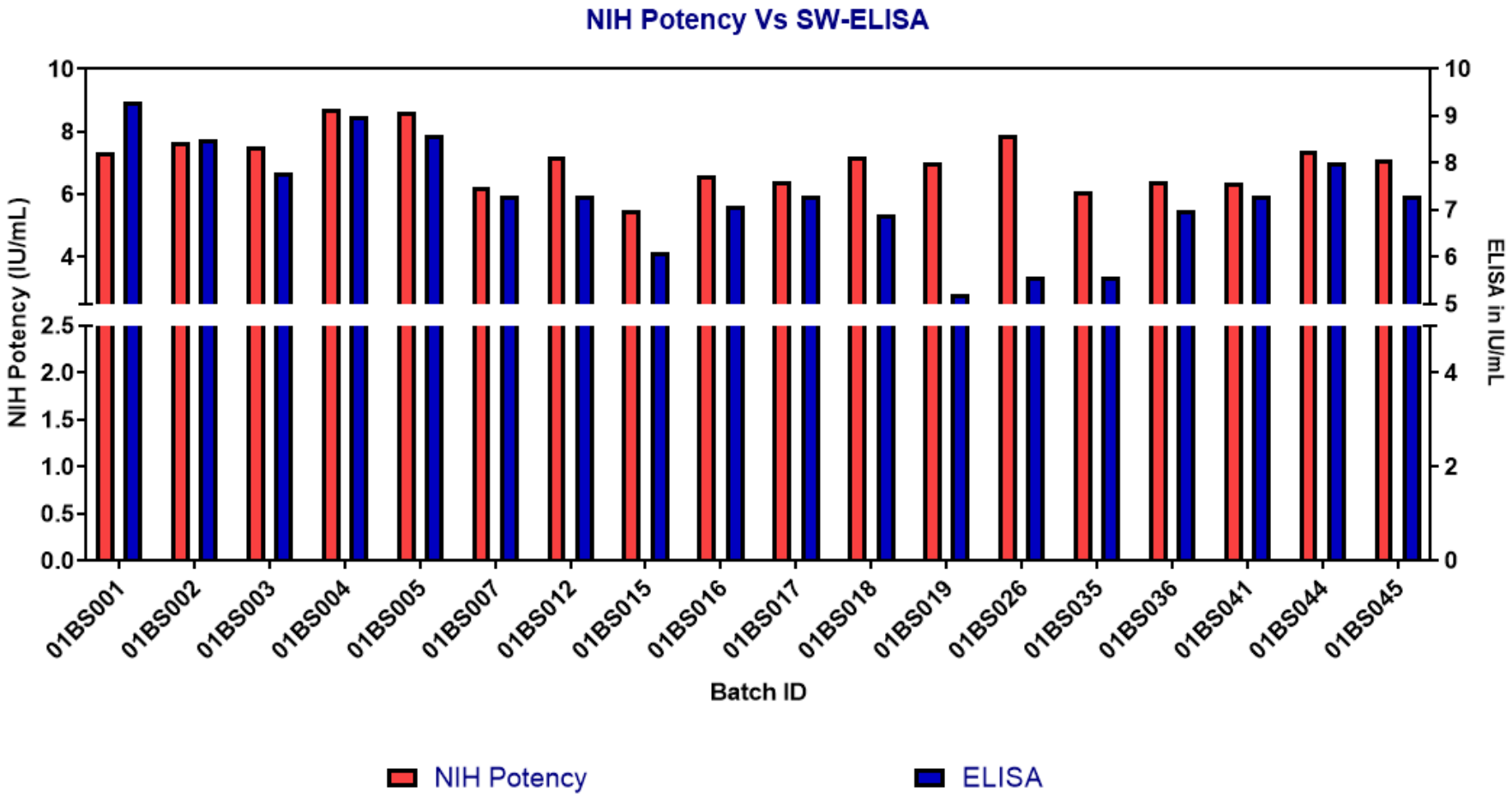
❑ 18 Batch Data of NIH Potency Vs SW-ELISA



❑ Cost of Animal Potency

Rabies Vaccine Testing								
	Method	No of Animals used per Batch	Cost per animal (Approx. on the lower side) in Rs.	Husbandry cost for 28 Days per animal in RS.	Total cost for a 28 day batch release study in Rs.	No of Rabies batches taken in 2017-18	Total Animal Cost for releasing the batches in Rs.	Remarks
Current Method	NIH Animal Potency	136	200	300	68,000	60	40,80,000	Handling of challenge virus, Facility to do challenge study

Total No. of Animals Approximately Consumed- ~5000- 8000 Nos.

❑ Cost of Sandwich ELISA

	Method	Time for Test	Cost of the Assay per plate (Approx. for testing 5 batches) in Rs.	No of Rabies batches taken in 2017-18	Total Cost for testing 60 batches in Rs.
Alternate Method	In-house Developed Sandwich ELISA (EDQM harmonizing to approve)	1 Day	300	60	18,000

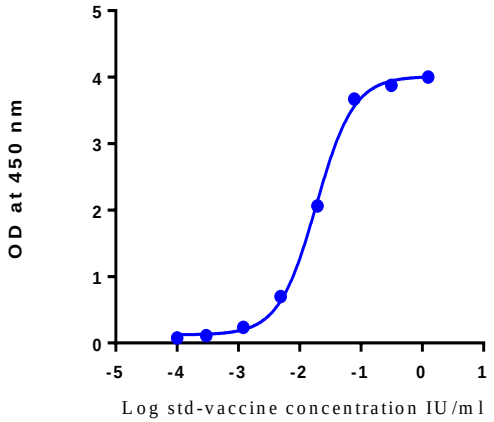
No Animal Usage

❑ Zydus approach to Development of *in-vitro* potency assay G-Protein ELISA method for Rabies vaccine testing

- In-house highly characterized mAbs binding to the G protein of Rabies antigen

1. M777-16-3 **(IgG1)** binds to **Site II**
2. 62-71-3 **(IgG2b)** binds to **Site III**

- Developed sandwich ELISA and made the standard curve
- Assay range is from **1.25IU/mL to 0.01IU/mL**
- Screened 18 batches of Rabies vaccine by ELISA method



- Correlation between NIH and ELISA was studies
- **More validation under QC is under progress to under stand the potent and low potent batches**

❑ Questions to be answered for Replacing the in-vivo NIH with in-vitro assay

- Are all manufacturers interested in moving from in-vivo NIH potency to in-vitro (ELISA) method?
- Are the critical reagent available to implement the assay?
- Method validation/correlation for the assay performed?
- Are enough lots tested or Is it possible to get more manufactures share the final lot for validating the Assay?
- Can a harmonized training provided to all manufacturers?

❑ Conclusion

- Zydus Cadila as one of the leading vaccine manufacturers of Human Rabies vaccine is interested to collaborate and validate the assay platform through DCVMN network program
- **Further Validation of method is in Progress**

Thank You