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Corresponding Author:	Noel Tordo Institut Pasteur Paris, FRANCE
Corresponding Author's Institution:	Institut Pasteur
Corresponding Author E-Mail:	noel.tordo@gmail.com
Order of Authors:	Noel Tordo
	Corinne Jallet
	Noël Tordo
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TITLE:

In Vitro ELISA Test to Evaluate Rabies Vaccine Potency

AUTHORS AND AFFILIATIONS:

Corinne Jallet[1], Noël Tordo[1,2]

[1]Unit Antiviral Strategies, OIE Reference Laboratories for Rift Valley Fever Virus & Crimean Congo Hemorrhagic Fever Virus, Institut Pasteur, Paris, France

[2]Institut Pasteur de Guinée, Conakry, Guinea

Corresponding Author:

Noël Tordo (ntordo@pasteur.fr)

Email Addresses of Co-author:

Corinne Jallet (cjallet@pasteur.fr)

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SUMMARY

Here we describe an indirect ELISA sandwich immunocapture to determine the immunogenic glycoprotein contents in rabies vaccines. This test uses a neutralizing Monoclonal Antibody (mAb-D1) recognizing glycoprotein trimers. It is an alternative to the in vivo NIH test to follow the consistency of vaccine potency during production.

ABSTRACT

The growing global concern for the animal welfare is encouraging manufacturers and the National Control Laboratories (OMCLs) to follow the 3Rs strategy for the Replacement, Reduction, and Refinement of the laboratory animal testing. The development of in vitro approaches is recommended at the WHO and European levels as alternatives to the NIH test for evaluating the rabies vaccine potency. At the surface of the rabies virus (RABV) particle, trimers of glycoprotein constitute the major immunogen to induce Viral Neutralizing Antibodies (VNAbs). An ELISA test, where Neutralizing Monoclonal Antibodies (mAb-D1) recognize the trimeric form of the glycoprotein, has been developed to determine the contents of the native folded trimeric glycoprotein along with the production of the vaccine batches. This in vitro potency test demonstrated a good concordance with the NIH test and has been found suitable in collaborative trials by RABV vaccine manufacturers and OMCLs. Avoidance of animal use is an achievable objective in the near future.

The method presented is based on an indirect ELISA sandwich immunocapture using the mAb-D1 which recognizes the antigenic sites III (aa 330 to 338) of the trimeric RABV glycoprotein, i.e., the immunogenic RABV antigen. mAb-D1 is used for both coating and detection of glycoprotein trimers present in the vaccine batch. Since the epitope is recognized because of its

conformational properties, the potentially denatured glycoprotein (less immunogenic) cannot be captured and detected by the mAb-D1. The vaccine to be tested is incubated in a plate sensitized with the mAb-D1. Bound trimeric RABV glycoproteins are identified by adding the mAb-D1 again, labeled with peroxidase and then revealed in the presence of substrate and chromogen. Comparison of the absorbance measured for the tested vaccine and the reference vaccine allows for the determination of the immunogenic glycoprotein content.

INTRODUCTION

Since more than 50 years, the NIH test[1] is used as a gold standard method to evaluate the rabies vaccine potency before the batch release. This test consists of an intraperitoneal immunization of groups of mice with the vaccine to be tested followed by an intra-cerebral (IC) challenge 14 days later with the Challenge Virus Standard (CVS) strain of rabies virus (RABV). The potency is evaluated from the proportion of mice surviving the IC challenge. Although WHO[2] and European Pharmacopeia[3] still require the NIH test for assessing the vaccine potency, this test suffers several hurdles: results are highly variable[4]; infectious RABV is used during the challenge and this requires both technical skill and strict biosafety measures; large numbers of animals are used, and the severity of the challenge raises serious ethical concerns[5]. A less severe variation of this test has been developed: two weeks after the intra-peritoneal immunization, mice are not challenged by IC but bled and tested for the presence of specific RABV neutralizing antibodies (VNAbs) in their serum using an in vitro neutralization test. However, this test still requires sacrificing a large number of laboratory mice although it is already in use for the veterinary vaccines[6,7] and has been considered for human vaccines[8].

As of now, both International[9] and European[10] recommendations encourage manufacturers and National Control Laboratories (Official Medicine Control Laboratories - OMCLs) to implement the Replacement, Reduction, and Refinement of laboratory animal testing, referred as the 3Rs strategy. European Directive 2010/63/EU (in force since 2013/01/01) related to the protection and welfare of animals has also reinforced the constraints for vaccine manufacturers and laboratories involved in the Quality Control of rabies vaccines as well as in rabies research[11]. As a result, the development, validation, and use of alternative in vitro approaches have now become a priority. These are not only ethically sound but can also reduce the batch testing costs and shorten the time for results to hours instead of weeks[3].

At the surface of the RABV particle, the glycoprotein adopts a trimeric form[12-16]. In rabies vaccine, this native trimeric form constitutes the major immunogen inducing VNAbs[17] while the monomeric, soluble or denatured glycoproteins are poorly immunogenic[18,19]. Thus, the preservation of trimers of the glycoprotein along the vaccine production process is a good indicator for the preservation of an optimal immunogenic potential. Several immunochemical methods, such as the antibody-binding-test[20,21], the single radial immunodiffusion (SRD) test[22] and the ELISA test[23-27] are recommended by the WHO Technical Report Series[2] and the European monograph[3] to quantify the antigen content in rabies vaccines. These are used by manufacturers to monitor the consistency of vaccine production and by the OMCL to assess the consistent formulation of batches of human vaccines[28], even if the NIH test is still considered for the potency.

However, all these immunochemical methods are not equivalent. The SRD test requires a pre-treatment which may alter the membrane-anchored trimers and result in a soluble or denatured form of the glycoprotein[22,29]. Hence, SRD is not much efficient in discriminating between immunogenic and non-immunogenic glycoproteins resulting in an imperfect appraisal of the immunogenicity of a vaccine lot. By contrast, the ELISA test is more sensitive[22], preserves the native structure of the glycoprotein, and is more appropriate to determine the content of the natively folded trimers of glycoprotein. The ELISA test can use either rabbit polyclonal or mouse monoclonal anti-glycoprotein antibodies purified or concentrated with ammonium sulfate. Studies have demonstrated good concordance between the NIH test and the antigen content evaluated by ELISA in vaccines and concluded that ELISA methods are suitable for the in vitro potency test. This advocates that ELISA tests might at least supplement or even replace the NIH test[4,26,27,30-33]. Today, the European Pharmacopoeia recommends the use of validated serological or immunochemical assays as alternatives to the NIH test[3]. The complete avoidance of animal use for vaccine potency has become a realistic perspective.

The method presented below is based on an indirect ELISA sandwich immunocapture using a mouse monoclonal antibody clone (mAb-D1) which recognizes the antigenic sites III (aa 330 to 338) of the trimeric RABV glycoprotein[15,34]. This method was developed initially at the Institut Pasteur[26,30] then optimized and validated by the *Agence Nationale de Sécurité du Médicament et des produits de santé* (ANSM) laboratory, i.e., the French OMCL[4,33]. The mAb-D1 is used both for sensitizing the plate and subsequently for detecting the captured antigen. This allows for the specific quantification of the glycoprotein trimers, i.e., the immunogenic RABV antigen. The mAb-D1 used for the detection is labeled with peroxidase, which is revealed in the presence of the substrate and chromogen. Comparison of the absorbance measured for the tested vaccine and the reference vaccine allows for the determination of the immunogenic glycoprotein content. It is of note that the same type of assay can be applied for different mAbs recognizing different antigenic sites of the RABV glycoprotein[35]. The method to obtain and purify or concentrate with ammonium sulfate anti-glycoprotein polyclonal rabbit immunoglobulins G (IgG) or monoclonal mouse globulins have been extensively described previously[36] along with the method to conjugate antibodies with peroxidase[37].

PROTOCOL

1. Security precautions

NOTE: This method is applicable to both live RABV and inactivated vaccine.

1.1. Use good Laboratory Practice and Safety procedures.

1.2. Wear adequate Personal Protection Equipment (PPE) including disposable coat, gloves, mask, glasses, etc.

1.3. When the live virus is titrated, use a class II biological safety cabinet.

1.3.1. Consider any material in contact with the samples (reagents, washing solutions, etc.) as infectious material.

1.3.2. Treat the contaminated material by immersing in the bleach solution (5% of sodium hypochlorite) for 30 min for decontamination.

1.4. Handle chemicals in accordance with the Good Laboratory Practice.

2. Preparation

2.1. Use analytical grade reagents where ever possible.

2.2. Prepare fresh solutions of the coating buffer/carbonate buffer, passivation buffer, diluent and citrate buffer (**Table 1**), filter through 0.45 or 0.22 µm filters and store at 4 °C for one day prior to the use to preserve their analytical purity.

2.3. Allow reagents to reach to the room temperature (+18 °C to +25 °C) 30 min before the use and homogenize by gentle mixing prior to the use.

3. Microplate sensitization

NOTE: Use 96 well adsorption immunoassay plates which are optimized to bind high amounts of Immunoglobulins (e.g., see **Table of Materials**).

3.1 To each well, add 200 µL of the monoclonal antibody (mAb-D1) diluted in the carbonate buffer.

NOTE: An optimal concentration of about 1 µg/mL has been experimentally determined and corresponds to an approximate 1/2000 dilution of the purified mAb-D1. This recommended concentration is indicated for each mAb-D1 batch and must be periodically verified with the positive control.

3.2. Cover the plate with an adhesive film and incubate the microplate for 3 h at 37 °C in a humidified atmosphere.

3.3. Carefully aspirate and transfer the well content into a recipient containing 5% sodium hypochlorite solution.

3.4 Invert the microplate and let it dry on an adsorbent paper at room temperature for 5 min.

4. Microplate passivation

4.1. To each well, add 300 µL of the passivation buffer.

4.2. Cover the plate with an adhesive film and incubate for 30 min at 37 °C.

4.3. Aspirate carefully and transfer the well content into a recipient containing 5% sodium hypochlorite solution.

4.4. Invert the microplate and let it dry on an adsorbent paper at room temperature for 1 min.

NOTE: The microplate can be immediately used or stored sealed at -20 °C for up to 3 months until use.

5. ELISA assay

NOTE: For establishing the control curve of the reference vaccine, Step 5.3 is not required; to titrate the tested vaccine all Steps 5.1 to 5.6 are necessary.

5.1. Washing of the sensitized microplate

5.1.1. To each well, add 300 µL of the washing buffer.

5.1.2. Aspirate carefully and transfer the well content into a recipient containing 5% sodium hypochlorite solution.

5.1.3. Repeat steps 5.1.1 and 5.1.2 five more times to extensively wash the sensitized plate.

5.1.4. Invert the microplate and let it dry on an adsorbent paper at room temperature for 1 min.

5.2. Dilutions of the reference vaccine for the control curve

5.2.1. Reconstitute the reference vaccine (validation antigen Lot 09) in 1 mL of distilled water corresponding to a concentration of 10 µg/mL of rabies virus glycoprotein.

5.2.2. Prepare a ten-fold dilution of the reconstituted reference vaccine in the diluent to reach 1 µg/mL of rabies virus glycoprotein.

5.2.3. Prepare 6 serial two-fold dilutions of this reference vaccine in the diluent as indicated in **Table 1**.

5.2.4. Distribute 200 µL of the diluent in duplicate (wells 1H/2H) to serve as a blank control.

5.2.5. Distribute 200 µL per well of each reference vaccine dilution in duplicate (wells G1/G2 to A1/A2).

5.3. Dilutions of the tested vaccine for its titration

5.3.1. Prepare a ten-fold dilution of the tested vaccine in the diluent.

5.3.2. Prepare 7 two-fold serial dilutions of the tested vaccine in diluent as indicated in **Table 2**.

5.3.3. Distribute 200 µL per well of each tested vaccine dilution in duplicate (wells H3/H4 to A3/A4).

5.4. Incubation/Washing of the ELISA plate

5.4.1. Cover the microplate with an adhesive film and incubate for 1 h at 37 °C.

5.4.2. Remove the film, aspirate carefully and transfer the content of each well into a recipient containing 5% sodium hypochlorite solution.

5.4.3. To each well add 300 µL of washing buffer.

5.4.4. Aspirate carefully and transfer the content of each well into a recipient containing 5% sodium hypochlorite solution.

5.4.5. Repeat steps 5.4.3 and 5.4.4 five times to remove the unbound antigen and conserve the G protein trimers bound to the coated antibody (mAb D1).

5.4.4. Invert the microplate and let it dry on an adsorbent paper at room temperature for 1 min.

5.5. Binding of the peroxidase conjugated mAb-D1

5.5.1. Distribute 200 µL per well of the recommended dilution (1/2000) of peroxidase-labeled mAb-D1 in diluent (approximate concentration of 1µg/mL). A recommended concentration is indicated for each mAb-D1 batch and has to be periodically verified with the positive control.

5.5.2. Cover the microplate with an adhesive film and incubate for 1 h at 37 °C.

5.5.3. Remove the film, aspirate carefully and transfer the content of each well into a recipient containing 5% sodium hypochlorite solution.

5.5.4. Add to each well, 300 µL of the washing buffer.

5.5.5. Aspirate carefully and transfer the content of each well into a recipient containing 5% sodium hypochlorite solution.

5.5.6. Repeat steps 5.5.4 and 5.5.5 five times to remove unbound peroxidase-labeled antibody (mAb D1).

5.5.7. Invert the microplate and let it dry on an adsorbent paper at room temperature for 1 min.

5.6. Revelation using a substrate-chromogen

5.6.1. Distribute 200 µL per well of substrate-chromogen solution.

5.6.2. Seal the microplate with a film and incubate in the dark at room temperature for 30 min. A yellow-orange color develops the intensity of which is proportional to the amount of bound peroxidase-labeled antibody (mAb D1).

5.6.3. Stop the reaction by adding 50 µL of stopping solution per well.

5.6.4. Carefully wipe the bottom of the microplate and place it in a spectrophotometer to determine the optical density (OD) at 492 nm of all used wells: negative control (blank), reference vaccine and tested vaccine.

5.6.5. Collect OD data as .xls or .xlsx file format for analysis.

5.7. Draw the reference vaccine curve, the trimeric glycoprotein content, as a function of optical density (492 nm)

5.7.1. Calculate the mean OD at 492 nm for each duplicate at the different dilutions of the reference vaccine (wells G1/G2 to A1/A2 in **Table 2**)

5.7.2. Subtract the mean OD of the Blank (wells, H1/H2 in **Table 2**) from each calculated mean OD.

5.7.3. Plot the resulting OD values on the vertical axis (linear scale) and the corresponding concentration in glycoprotein trimers (ng/mL) on the horizontal axis (logarithmic scale).

5.7.4. Draw the reference curve by joining points (**Figure 1**).

REPRESENTATIVE RESULTS

In the following example the reference vaccine Lot 09, consisting of purified inactivated rabies virus particles (PV vaccine strain), is used. The glycoprotein trimers (10 µg/mL) content in this has been established after the determination of the total amount of viral proteins (BCA test) and evaluation of the percentage of the glycoprotein by SDS-polyacrylamide gel electrophoresis. Alternatively, a calibrated reference vaccine, e.g., the WHO 6th International Standard (IS) for Rabies Vaccine (NIBSC code: 07/162), can be used.

Table 3 shows the OD values (492 nm) for a typical experiment. Using these values, the reference vaccine curve was drawn by plotting (1) the mean OD (minus the mean OD of the blank) at the

different dilutions of the reference vaccine on the vertical axis (linear scale); (2) the concentration of the glycoprotein trimers (ng/mL) on the horizontal axis (logarithmic scale) (**Figure 1**).

The glycoprotein content of the tested vaccine is estimated by comparison to this reference vaccine curve. The evaluation is precise for the dilution of the tested vaccine giving a mean OD value in the linear part of the reference vaccine curve. In the presented experiment (**Figure 1**), the linear part is from about 1 to 2 OD **(Table 3).**

The dilution 1/40 of the tested vaccine, with a mean OD for duplicate samples of 1.534, is then appropriate for further evaluation. When this OD is plotted horizontally up to the point of intersection with the reference vaccine curve, the vertical projection on the x-axis corresponds to 500 ng/mL of glycoprotein. Considering the dilution, the tested vaccine contains

40 x 500 ng/mL = 20 µg/mL of trimeric glycoprotein.

Currently, there is no ELISA international unit for RABV glycoprotein content. However, the in vivo potency of the reference vaccine, in international units (IU/mL), has been established using the NIH test in comparison to the 6th WHO International Standard (NIBSC code: 07/162)[4]. Consequently, the comparison of the mean ODs between the reference and the tested vaccine not only allows the measurement of the trimeric glycoprotein amount of the tested vaccine but also evaluates the in vitro potency estimated in Equivalent International Unit (EIU/mL).

Using the ELISA method described here, the French OMCL (ANSM) has monitored the glycoprotein content of more than 1000 batches of human rabies vaccine to be released in the market and has compared to the NIH test performed at the manufacturer's site[4]. The high variability of the NIH test, due to the heterogeneity in mice strain and challenge procedure[38], prevented a statistical correlation between the two tests. However, a concordance in the profile of results and the same pass/fail conclusions were obtained using in vitro and in vivo assays[4]. This concordance confirms that the native trimers of the glycoprotein recognized by the mAb-D1[34,39] constitute the main rabies virus immunogen inducing VNAbs during vaccination[17]. These VNAbs are able to protect mice from the intra-cerebral challenge of the NIH test. In conclusion, the in vitro appraisal of the rabies glycoprotein content is an attractive alternative to the NIH test evaluate rabies vaccine potency.

FIGURE AND TABLE LEGEND:

Table 1. Buffers used in the assay.

Table 2: Microplate plan for rabies glycoprotein titration assay and dilutions for the reference and tested vaccines used in the assay.

Table 3. Results obtained at OD_{492} nm for plotting the reference curve

Figure 1: Reference curve showing the trimeric glycoprotein content as the function of the optical density (492 nm) for the reference vaccine.

DISCUSSION

The epitope recognized by the mAb-D1 is located in the antigenic site III of the RABV glycoprotein which is not only immuno-dominant for the induction of VNAbs but also involved in neurovirulence, pathogenicity[40,41] and receptor recognition[42]. There is another important antigenic site along the glycoprotein, site II[43], against which several MAbs have been isolated such as mAb-WI-1112[35]. These can also be used in the similar type of experiments.

One limitation of this in vitro method by ELISA resides in the necessary conservation of the epitope recognized by the used mAb on the rabies virus strain to be tested. Up to now, all the classical strains used for human rabies vaccines are recognized by the mAb-D1. The greater diversity of rabies strains used in animal vaccines may constitute a problem in the future. However, as mentioned earlier, the same assay can be applied using different mAbs recognizing different antigenic sites of the RABV glycoprotein for coating and detection. This will allow circumventing the problem[35].

Another sensitive point of this method, quantifying the trimeric form of the RABV glycoprotein, is the possible effect of pH and temperature on the reversible conformation conversion[14]. These parameters are taken into account in the proposed protocol.

In summary, quantification of a highly immunogenic epitope of correctly folded glycoprotein trimers by in vitro ELISA method appears as effective as the NIH test to measure the capacity of a vaccine batch to induce humoral immunity against rabies virus infection. In addition, the ELISA method can discriminate sub-potent vaccine lots, in quality or in quantity, from the potent ones[4,35]. The last step before proposing such an in vitro ELISA assay to replace the NIH test is the organization of an international collaborative study for its improvement and standardization.

A workshop of the Interagency Coordinating Committee on the Validation of Alternative Methods *(*ICCVAM*)* entitled International Workshop on Alternative Methods to Reduce, Refine, and Replace the Use of Animals in Vaccine Potency and Safety Testing (Ames, September 2010)[44], concluded that the NIH test should be replaced by an alternative in vitro test evaluating the vaccine immunogenicity and able to discriminate between potent and sub-potent batches[4]. Another workshop of the European Partnership for Alternatives to Animal Testing (EPAA) in 2012[45] decided that a standardized sandwich ELISA calibrated against the current international rabies reference standard would be an ideal alternative for the rabies vaccine potency testing. Following, an international collaborative pre-validation study, which included both manufacturers and regulatory bodies, compared various ELISA designs used by the manufacturers and their National Control Laboratories for batch release for their ability to discriminate sub-potent from potent batches from different vaccine brands[35]. An ELISA design combining mAb-D1 (antigenic site III) and a different mAb-WI-1112 (antigenic site II) was proposed by the European Directorate for the Quality of Medicines & HealthCare (EDQM) for a forthcoming international collaborative study under the umbrella of the Biological

Standardization Program (BSP). This shows (1) that several combinations of mAbs for microplate coating and detection can be used as an in vitro alternative to the in vivo NIH test and (2) that these combinations can be dependent on the RABV vaccine strain to be tested.

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DISCLOSURES

The authors have nothing to disclose.

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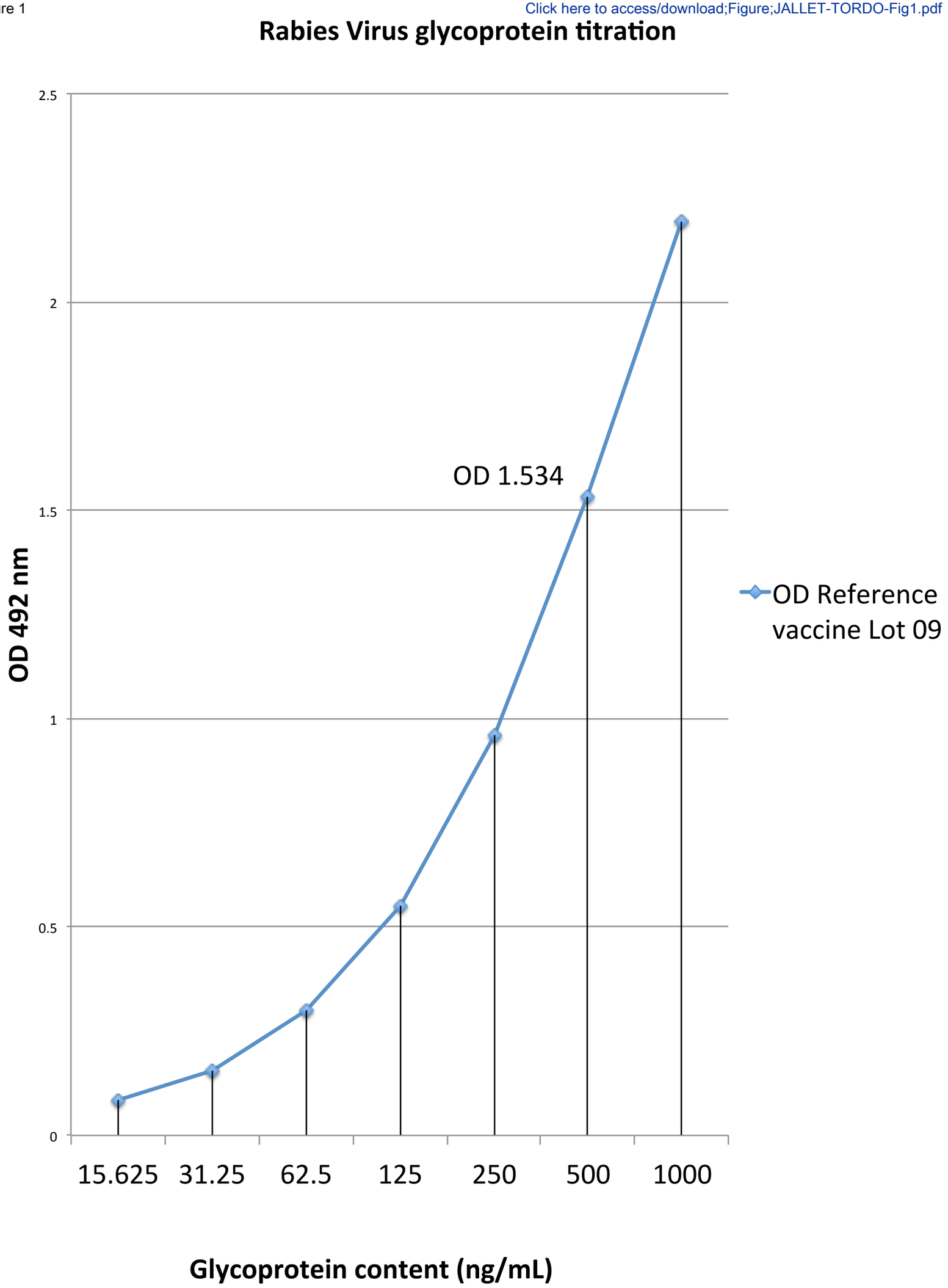
Figure 1



Buffers and reagents	
Coating buffer (*Carbonate buffer 50mM pH=9.6*)	Add Sodium bicarbonate
Passivation buffer	0.3% Bovine
10x Phosphate buffered saline pH=7 (*PBS 10x*)	NaCl 80 g, K(distilled wat(
Washing buffer	0.05% Twee(
Diluent	0.5% Bovine
Citrate buffer pH-5.6 (for peroxidase substrate)	11.67 g Tri-s(acid-1H20 ir
Substrate-chromogen solution	50 mg Ortho
Stopping solution (4N sulfuric acid)	10 ml H2SO4 be carried ou

Preparation
carbonate 50 mM (Na_2CO_3-$10H_2O$) to Sodium 50 mM ($NaHCO_3$) until the desired pH (about 1/10
Serum Albumin (BSA, fraction V), 5% sucrose in
Cl 2 g, Na_2PO_4-$12H_2O$ 11.33 g, KH_2PO_4 2g in 1L of er. Adjust pH=7 with 4N NaOH
n in 1x PBS
Serum Albumin (Fraction V), 0.05% Tween in 1x PBS
odium citrate-$2H_2O$ ($Na_3C_6H_5O_7$-$2H_20$), 2.17 g Citric 1 1L of distilled water
-phenylene diamine tablet, 0.1% Hydrogen peroxide
36N in 80 ml cooled distilled water. Dilution must ut in an ice bath

Table 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	Ref. Vaccine 1/640	Ref. Vaccine 1/640	Tested Vaccine 1/1280	Tested Vaccine 1/1280								
B	Ref. Vaccine 1/320	Ref. Vaccine 1/320	Tested Vaccine 1/640	Tested Vaccine 1/640								
C	Ref. Vaccine 1/160	Ref. Vaccine 1/160	Tested Vaccine 1/320	Tested Vaccine 1/320								
D	Ref. Vaccine 1/10	Ref. Vaccine 1/10	Tested Vaccine 1/160	Tested Vaccine 1/160								
E	Ref. Vaccine 1/40	Ref. Vaccine 1/40	Tested Vaccine 1/80	Tested Vaccine 1/80								
F	Ref. Vaccine 1/20	Ref. Vaccine 1/20	Tested Vaccine 1/40	Tested Vaccine 1/40								
G	Ref. Vaccine 1/10	Ref. Vaccine 1/10	Tested Vaccine 1/20	Tested Vaccine 1/20								
H	Blank	Blank	Tested Vaccine 1/10	Tested Vaccine 1/10								

Reference vaccine Lot 09 dilution	Concentration (ng/mL)
1/640	15.625
1/320	31.25
1/160	62.5
1/80	125
1/40	250
1/20	500
1/10	1000

Table 3

Reference vaccine Lot 09 dilution	Concentration (ng/mL)	Tested vaccine dilution	OD column 1	OD column 2	OD Mean	Reference OD mean- Blank OD mean
1/640	15.625	**1/1280**	0.17	0.16	0.165	**0.0825**
1/320	31.25	**1/640**	0.233	0.238	0.2355	**0.153**
1/160	62.5	**1/320**	0.378	0.387	0.3825	**0.3**
1/80	125	**1/160**	0.619	0.644	0.6315	**0.549**
1/40	250	**1/80**	1.006	1.077	1.0415	**0.959**
1/20	500	**1/40**	1.559	1.674	1.6165	**1.534**
1/10	1000	**1/20**	2.245	2.307	2.276	**2.1935**
Blank	Blank	**1/10**	0.078	0.087	0.0825	

Table of Materials

Click here to access/download;Table of Materials;JALLET-TORDO-Materials.xls

Equipment	Company	Catalog Number	Comments/Description
Adsorption MAXISORP Flat Bottom	ThermoFisher Scientific	15036	
Class II Biological Safety Cabinet	ThermoFisher Scientific	10445753	if titrating live virus
Clear Flat-Bottom Immuno Nonsterile 96-Well Plates, 400 µL, MAXISORP	ThermoFisher Scientific	439454	good for binding to the loaded antibody
Equip Labo Polypropylene Laboratory Fume Hood	ThermoFisher Scientific	12576606	for the preparation of sulfuric acid
Immunology Plate Strong Adsorption MAXISORP Flat Bottom Well F96	Dutscher	55303	good for binding to the loaded antibody
Microplate single mode reader Sunrise	TECAN		
Microplate shaker-incubator	Dutscher	441504	
Microplate washer Wellwash	ThermoFisher Scientific	5165000	
Multichannel pipette (30-300 µL) 12	ThermoFisher Scientific	4661180N	
Single Channel pipettes (Kit 2 : Finnpipettes F2 0.2-2 µL micro, 2-20 µL, 20-200 µL & 100-1000 µL)	ThermoFisher Scientific	4700880	
Well F96	ThermoFisher Scientific	VLB000D1	

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Author(s): CORINNE JALLET and NOËL TORDO (NT CORRESPONDING)

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CORRESPONDING AUTHOR

Name:	TORDO Noël
Department:	Antiviral Strategies Unit
Institution:	INSTITUT PASTEUR
Title:	Head of Unit
Signature:	INSTITUT PASTEUR Unité de Stratégies Antivirales Noël TORDO 25-28, rue du Docteur Roux 75724 Paris Cedex 15 Tél. +33 (0)1 40 61 31 34 Fax : +33 (0)1 40 61 31 45
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Rebutal Letter

Editorial comments:

Changes to be made by the Author(s):

1.Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

We have done our best as not native English speaking persons. Last time one of mine manuscripts was corrected by native English speaking person before sending, I had the same remark that you are doing, both for spelling, grammar and style issues…! May be each Editor should define his Editing rules/language and edit manuscripts accordingly. Several do for the quality and the homogeneity of their publications…

2. Please format the manuscript as: paragraph: Indentation 0 for both left and right and special: none, Line spacings: single. Please leave a single line space between each step, substep and note in the protocol section.

Done, I hope the manuscript is soft to see as it stands

3. Please reword the title to remove colon or any punctuation marks and make it crisp to reflect the protocol described in the manuscript.

Done

4. Please provide at least 6 keywords or phrases.

We have added one to make 6

5. Please rephrase the Short Abstract/Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: “Here, we present a protocol to …”

Done (48 words).

6. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please consider moving lines 107-122 to the introduction section.

Small paragraphs have been done all over the protocol and results sections. The lines 107-122 have been moved in the introduction section.

7. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.) with all specific details in a stepwise manner. The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”

The imperative tense has been used. Note have been added when necessary

8. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes.

This has been modified

9. The Protocol should contain only action items that direct the reader to do something.

Done

10. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed?

We have added more details to describe the actions that are now completely described (including some repetitions)

11. 1.1: What is the appropriate dilution and how is it determined? Citation for previous tests?

« Appropriate » has been replaced by « recommended » and the text has been modified in lines 147-150 to explain how the dilution was calibrated in our lab in order to obtain a sigmoid reference curve with a linear part with the optimal signal/background ratio.

12. In which step did you add the serum? Where did you add the virus? Please write exactly how you perform your experiment, detailing everything in a step wise fashion.

Steps have been well separated now : Step 3 (and sub-steps) for adding the mAb-D1, Step 5 (and sub-steps) for adding the vaccine

13. 3.3: where did you get the vaccine from? What is the difference between reference vaccine and the one described in 3.4? Please detail the dilutions used in your experiment. Please do not generalize and be specific to your experiment throughout the protocol.

The reference vaccine we are classically using is “Lot 09” as indicated in lines 179-180. To clarify the point, we have added a specific note at the beginning of the “Results” (lines 238-243) explaining that this vaccine has been calibrated with the WHO 6th International Standard (IS) for Rabies Vaccine (NIBSC code: 07/162). The latter can also be use used directly as the reference vaccine.

14. Please remove the embedded Table of buffer and reagents from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.

Done

15. Please provide the result for the glycoprotein content of the test vaccine as determined by your experiment.

The results for the tested vaccine are shown in representative results, the dilution 1/40 which is giving an OD 1,534, well fitted in the linear part of the reference curve is taken as an example to calculate the G protein titre of the tested vaccine, namely 20 µg/ml (lines 259-268).

16. Once done please highlight 2.75 pages of the protocol including headings and spacing in yellow for filming purpose. The highlighted steps should form a cohesive narrative with a logical flow from one highlighted step to the next. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Done

17. Please alphabetically sort the materials table.

Done

18. Please include a title and a description of each figure and/or table. All figures and/or tables showing data must include measurement definitions, scale bars, and error bars (if applicable). Please include all the Figure Legends together at the end of the Representative Results in the manuscript text.

Done

Reviewer #1:

Manuscript Summary:

This article focused on the development of indirect ELISA method to evaluate rabies vaccine potency. The core of this method is based on two hypotheses: 1) the neutralizing monoclonal antibody (mAb-D1) used for both coating and detection can recognize specific and conservative antigenic site existing in the surface of rabies virus glycoprotein trimer, and 2) the content of rabies virus glycoprotein trimer can be positively correlated to vaccine potency. I have no doubt about them as several published studies have already confirmed them. In my opinion, the indirect ELISA method is very useful in the evaluation of vaccine stability and may be used as supplement of NIH test in potency evaluation of rabies vaccine. However, few points need to be revised or supplemented before acceptance for publication.

Minor Concerns:

1. It should be clarified that all buffers should be filtered through sterile filter system (0.45 or 0.22 um) as all regents may probably be at the level of analytical purity.

This has been added in lines 137-139

2. On page 9, in the part of Passivation buffer, does BSA mean BSA (Fraction V)?

Yes, this has been added in lines 158-159

3. It would be better to supplement some key points or tips for the protocol, such as the possible effect of pH and temperature on the conformation conversion of rabies virus glycoprotein, and therefore the robustness of this protocol.

A sentence has been added in the discussion to clarify this point, lines 303-305

Reviewer #2:

Manuscript Summary:

The authors detailed the protocol of an ELISA to determine the content of the trimeric form of rabies virus glycoprotein. This protein is known to be responsible for the synthesis of neutralizing rabies virus antibodies, and also is involved in the pathogenicity of the virus and its attachment to the receptor. The trimeric form the glycoprotein is more immunogenic that the monomer form. Therefore estimation of the immunogenic form is of paramount importance in rabies vaccine production.

Major Concerns:

We think that the title of the manuscript is not adequate; it is premature and not correct to say that "in vitro test of rabies vaccine potency is ready to replace the NIH test". Morgeaux et al. (2017) (Vaccine. 2017 Feb 7; 35(6):966-971) have shown that the ELISA described by the authors does not give the correct titer of the rabies vaccine produced using different rabies virus strains. Morgeaux et al. recommend to conduct an international collaborative study using two different mAbs (one for coating and the other of detection); to set up an ELISA test that can replace the NIH test. Therefore I recommend that the authors modify the title of the manuscript, and modify its content accordingly

It is clear that using monoclonal antibody(ies) for Elisa offer(s) the risk of having one vaccine escaping to the recognition due to the Mab' specificity. This is the reason why we have written in lines 115-117: "It is of note that the same assay can be applied using different mAbs recognizing different antigenic sites of the RABV glycoprotein[35]". We have also recalled this possible limit in the discussion section (lines 297-302). Finally, the Morgeaux's publication cited by the reviewer was already mentioned in the text (Ref 35) in several parts including the "Perspective Section". One author of the present manuscript was co-authors of this publication which concluded that an ELISA design combining mAb-D1 (antigenic site III ; for revelation) and a different mAb-WI-1112 (antigenic site II ; for coating) was better than the D1/D1 presented in the present protocol. This has been specifically mentioned in line 324-333. However, (1) the present protocol, as indicated, can be adapted to various mAbs and (2) the D1/D1 system is successively used since decades by many companies, including those participating to the Morgeaux's trial. We are not claiming that it is the only possible cocktail as written in the text but D1, being used for capture or for revelation or for both, is the only MAbs so far demonstrated to recognise the trimeric form of G, which is the immunogenic one (see refs 15 and 34).

Page 5/lines 131-132: the authors mean "and covered with sealer sheet" instead of "or covered …"

Thank you very much, we have changed all over the text

Page 8/lines 180-184: please explain how to convert from IU/ml to EIU/ml

When **the WHO 6th International Standard (IS) for Rabies Vaccine (NIBSC code: 07/162),** calibrated in UI/ml by *in vivo* testing, is used to test *in vitro* potency, the results are expressed as equivalent International Units par ml (EIU/mL). Here, the reference vaccine Lot 09 is calibrated in µg/mL.

Page 9: Please indicate for all buffers and reagents the storage conditions and their shelf life

We have added a paragraph in section 2 (preparation) to deal globally with this point (lines 137-141)

Page 9/lines 194-195: Please indicate the exact amount of each solution to mix to prepare the carbonate buffer for coating

This has been specified in the table “materials”

Reviewer #3:

Manuscript Summary:

Conventional rabies vaccine potency testing still rests on mouse inoculation and challenge experiments. With regard of increasing concerns for animal welfare, alternative in vitro methods are recommended. This manuscripts described an ELISA based method to quantify the amount of trimeric rabies virus glycoprotein complexes in order to assess vaccine potency. Because a monoclonal antibody was used that only recognizes an epitope in the trimeric RABV, which is most likely a conformational epitope, the authors conclude that the immunogenic fraction of G protein in the vaccine is selectively detected, whereas denatured or monomeric G are not detected. Comparison of obtained results with vaccine potency detected by conventional in vivo NIH potency test (Fig. 2) indeed revealed a correlation of determined G levels with potency, thus making the provided protocol an important for the future use of such assays in rabies vaccine potency testing.

Overall, the manuscript is well written and in most cases the described steps are easy to follow. JoVE style has not been addressed in all parts of the manuscript (e.g. 1st para in protocol section, list of buffers on pages 9 and 10) and revision according to the specific requirements is recommended.

Major Concerns:

With regard to the structure of the manuscript, it is puzzling that the protocol starts with microplate sensitization although exact quantification of the glycoprotein content in a reference vaccine (as shown in fig. 1 of expected results) is crucial for the whole assay. In order to allow readers to use the described protocol, I suggest to add a step by step description of rabies virus glycoprotein quantification in the reference vaccine as a first point of the protocol with a standard curve as shown in figure 1 as an exemplary result. A second figure could show results from the ELISA related to the previously determined standard curve and comparison to in vivo NIH potency testing as shown in the recent figure 2 could be added as a third figure to highlight the correlation of the two tests, although this is not part of the provided protocol.

We thank the reviewer for this excellent suggestion and we have modified the manuscript accordingly. Only the last request about the correlation between the *in vivo* and the i*n vitro* test cannot be satisfied. Because of the large variability of the NIH test (ref 38) we can claim only concordance and not correlation between both test, as explained in lines 282-286 and ref 4.

In addition to the above comments about general structure of the manuscript, there are some further points to be addressed: It is not clear whether the protocol is worthwhile for all licensed rabies vaccines. This is an important point, since there can be a difference between vector vaccine expression only G (not in vector virus particle), attenuated live vaccines (G in particle and expression of G after infection of host cells) and inactivated rabies virus particles (multiple G copies on the surface). In the latter two cases, the argumentation in line 49ff that the "potentially denatured monomers of glycoprotein (with only one binding site available) cannot be captured and detected with the same mAB-D1" is not plausible. Given that a virus particle has multiple G copies on the surface, also in case of some "denaturation" multiple monoclonal antibodies may bind to the particles. It may be more important that the mAB-D1 recognizes a conformational

epitope only present in the trimer. Any indications for that? If live vaccines are tested, biosafety requirements and inactivation procedures prior to ELISA testing should be explained.

We thank the reviewer for this other clever remark. As explained earlier in the rebuttal, we cannot exclude that a different vaccine strain will escape to the mAb-D1, but this is true for all mAbs. Up to now, D1 recognizes the trimers of all human vaccine tested so far (PV, PM-derived vaccines). It is true that the main advantage of D1 is to recognize the trimers and Ref 15, working with purified protein (which may also be a potential vaccine) clearly demonstrate that DI used in capture and revelation was exclusively recognizing trimers of G. This being said, this is true that this is not crucial for a vaccine consisting of inactivated or attenuated viruses having many trimers on G on their surface and we have moderated the sentence accordingly in lines 49-52.

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Minor Concerns:

The method relies on the availability of mAB-D1 antibody but information about availability of the monoclonal antibody in a not conjugated and conjugated form is not provided (commercially available?). Is it possible to replace it by other antibodies?

Yes it is possible to use other antibodies and we have explained that extensively in the rebuttal. D1 is currently available through MTA at the Institut Pasteur (I have added the contact in the Material section) and will be commercialized soon through a company (discussion on their way).

line 150: it is emphasized that the lowest dilution must have a content about 1 µg/mL of rabies virus glycoprotein. Please provide information about how to assess before measurement by the method.

This concentration of the reference vaccine was calibrated when the ELISA with mAb-D1 was optimized in order to obtain an optimal signal/background ratio and a decent sigmoid curve.

line 157: what is an "approximate dilution" of the peroxidase-labelled mAb-D1. If no concentration is available, provide used dilution and highlight that it may vary dependent on the antibody or mAb-D1 batch.

We did a mistake and have replaced “approximate” by “recommended” dilution which is 1/2000. It is mentioned in lines 147-150 and 211-214.

Reviewer #4:

Manuscript Summary:

The manuscript outlines the methods of an indirect ELISA test to evaluate the potency of rabies vaccines during production using monoclonal antibodies which bind to immunogenic glycoproteins. The manuscript is well written throughout and reporting of a standardized method is highly relevant to the objectives of the 3R strategy. The argument for use of this test is clearly presented and the methods appear sound.

My comments are minor and are limited to phrasing and typos. Congratulations to the authors on a clear and useful manuscript.

Major Concerns:

None

Minor Concerns:

Line 59 – typo
Done

Line 64 – phrasing
Done

Line 94 – phrasing
Done

Line 113 – phrasing
Done

Line 155 – phrasing
I don't see the problem, sorry

Line 147 - 153 - Sections 3.2 to 3.4, it would be helpful to have a figure showing an annotated illustration of the microplate to refer to and avoid any ambiguity about these steps. I appreciate that this may become redundant once the video is available.
There is such a figure in the current new version of the manuscript. Thank you for the advise.

Line 170 – phrasing
Done

Line 254 – phrasing
Done