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POTENCY FOR BOVINE VACCINES CONTAINING
BOVINEHERPESVIRUS 1 (BOHV-1) CAUSAL AGENT OF THE
INFECTIOUSBOVINERHINOTRACHEITIS (IBR)



Guideline nº 1 - G.B.

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**POTENCY for bovine inactivated vaccines containing
Bovine herpesvirus 1 (BoHV-1) causal agent of the
Infectious Bovine Rhinotracheitis (IBR)**

1. INTRODUCTION

Bovine herpesvirus 1 (BoHV-1) is the etiological agent of the infectious bovine rhinotracheitis/ infectious pustularvulvovaginitis (IBR/IPV), disease of the domestic and wild cattle that causes a wide range of clinical signs including rhinotracheitis, vulvovaginitis, infectious pustularbalanoposthitis, conjunctivitis, abortion, enteritis and encephalitis (1, 2, 3).

After respiratory and genital infections, BoHV-1 becomes latent in the neural ganglia. Stress can induce reactivation of the latent infection and virus may be shed intermittently (1, 3).

Infection elicits an antibody response and a cell-mediated immune response within 7-10 days. Neutralizing antibodies may persist 5 years after infection, but re-stimulations (reactivation or vaccination) are needed to keep titers at detectable levels by viral neutralization technique. On the contrary, total antibodies, evaluated by ELISA, remain detectable for life (24).

In general, vaccines prevent the development of severe clinical symptoms and reduce the shedding of virus after infection, but they do not prevent infection. Several eradication campaigns with and without vaccination (mandatory and/or voluntary) are being carried out in Europe. Norway, Finland, Sweden, Austria, Denmark, Switzerland and various regions of Italy and Germany had eradicated the infection (1,7). In the rest of the world, infection is endemic and with high prevalence (8, 9, 10, 11).

Various attenuated and inactivated BoHV-1 vaccines are currently available in the region. In Argentina and Uruguay, the only authorized vaccines are inactivated ones. Vaccines contain strains of the virus, generally replicated during multiple passages in cell culture. Inactivated vaccines contain high levels of inactivated virus or portions of the virus particle (glycoproteins) supplemented with an adjuvant to stimulate an adequate immune response. Inactivated vaccines are administered intramuscularly or subcutaneously. Marker or DIVA (Differentiating Infected from Vaccinated Animals) vaccines are now available in various countries. These marker vaccines are based on deletion mutants or in a subunit of the virion, for example, glycoprotein E (12). This type of vaccines is used in Europe in countries that carry out eradication programs with vaccination campaigns (1,7). In endemic countries, intensive vaccination programs may reduce prevalence of infected animals (1).

For the approval of vaccines containing IBR, international control organisms (APHIS, USA; EMEA-CVMP, UE; OIE; VICH) (1, 2, 13, 14) require potency and efficacy assays in the target species, which imply vaccination and challenge of susceptible and seronegative bovines. Once the product is approved, the quality of each batch to be released must be controlled by a potency test that determines product immunogenicity in bovines or other laboratory animal model (*in vivo* test). Some agencies, for example the CVB, USDA allow *in vitro* potency tests using a parallel line assay and a validated reference vaccine [Title 9, Code of Federal Regulations (9 CFR) 113.8(a)(3)(ii)]. The *in vitro* potency test must be statistically validated and show an acceptable agreement when compared to the potency test in the target species. It is also strongly

desirable that the model be validated as a predictive tool of the degree of protection that the vaccine will provide against the viral shed in seronegative bovines. Due to the unavailability of seronegative bovines and the high cost of immunogenicity tests in the natural host, this potency and efficacy test cannot be carried out routinely in the target species. Therefore, it was decided to develop and validate a standardized test in laboratory animals (guinea pigs) that can assess potency of each vaccine batch, guaranteeing the presence of standardized and efficient products in the marketplace.

Regarding animal health, international organisms encourage the development of *in vitro* tests, to avoid and reduce to the minimum the use of animals for experimental control tests. In the particular case of these vaccines, simple or combined and inactivated, the application of these techniques is possible and it is worth exploring them. However, given the disadvantage that each formulation (group of inactivated antigens and adjuvant, vaccines in subunits and DNA vaccines) needs to be standardized, an *in vivo* test is still considered inevitable to assess potency of these products (17).

2. POTENCY CONTROL IN GUINEA PIGS: AIMS

In the guinea pig model, though it's an *in vivo* assay, the number of animals employed (n= 6 per vaccine and 4 witnesses/placebos) and the amount of blood extractions is reduced to the minimum. Guinea pigs, unlike other laboratory animals such as rats, have the advantage of being bigger in size, thus allowing paired serum sampling without risking their lives. Furthermore, with the volume of sample obtained, the quality of all viral antigens contained in polyvalent vaccines can be assessed. In some cases, such vaccines can contain 4 strains of these 5 agents: Bovine herpesvirus, bovine viral diarrhea virus, respiratory syncytial virus, parainfluenza virus type 3, bovine rotavirus. Some vaccines to prevent bovine neonatal diarrheas also include bovine coronavirus in their formulation.

Finally, serological evaluation is independent from the type of adjuvant (oil or water) and from the amount and quality of inactivated viruses contained in the formulation.

Guinea pig model: background

The trial assay for viral vaccines in guinea pig is based on the immunization of 6 guinea pigs in two doses of vaccine (with a 21 day interval), applied subcutaneously, of a volume equal to 1/5 the bovine dose. Animals are also kept under study during a minimum of 30 days. Serum samples are taken at the time of the first vaccine dose (0 days post-vaccination) and 9 days post-revaccination. Together with the assessment of unknown vaccine(s) (n=6), two groups of guinea pigs are included, one vaccinated with the reference vaccine of known potency (n=6) and the unvaccinated control group. Thirty days after the beginning of control, vaccinated animals are bled and a serological control by ELISA and viral neutralization is

performed. It is worth mentioning that guinea pigs are a BoHV-1 free species, so they are naturally seronegative to antibodies (Ab) against this viral agent.

Based on the results obtained since 2008, where all guinea pig serums obtained at the beginning of the test were negative, we can recommend annual control of the reproductive animals of the colony, eliminating likewise initial sampling of the animals, sampling the vaccinated and control groups only at the end of the test (30 dpv).

Validation of the guinea pig model for IBR strain, based on a linear regression analysis of the Ab titers determined by ELISA and viral neutralization (VN), indicated a dose-response relationship to the BoHV-1 antigen concentration in the vaccine in bovines and guinea pigs (dose-response assay). The guinea pig model was able to discriminate between vaccines containing 1 log₁₀ difference in its Ag concentration, both by ELISA and VN. Based on the results obtained in the dose-response curve, cut-offs or ranges of Ab titers anti-BoHV were estimated. These allow vaccines to be differentiated by the immunogenicity induced in guinea pigs and bovines. Two cut offs and three categories were established by ELISA (Table 1) and VN (Table 2). Finally, representative vaccines of each category were assessed in an experimental challenge test with IBR in seronegative bovines and the relation between Ab titer in guinea pigs and bovines and the degree of protection against infection was established (18).

SPECIES	VACCINE POTENCY ELISA		
	NON SATISFACTORY	SATISFACTORY	VERY SATISFACTORY
GUINEA PIG	$\bar{y} < 1.93$	$1.93 \leq \bar{y} < 3.02$	$3.02 \leq \bar{y}$
BOVINE	$\bar{Y} < 1.69$	$1.69 \leq \bar{Y} < 2.72$	$2.72 \leq \bar{Y}$

Table 1. Cut offs determined by ELISA expressed as the log₁₀ of the reciprocal of the analyzed serum dilution that results positive in the assay. Mean Ab titer of groups of 5 guinea pigs, evaluated 30 days post vaccination (dpv) and groups of 5 seronegative bovines evaluated 60 dpv. Bovines receive two doses of vaccine with a 30-day interval, following vaccine manufacturer's recommendations, and are sampled at 0 and 60 dpv. This latter point corresponded to the peak or plateau of Ab titers reached by aqueous or oil vaccines, respectively. Guinea pigs receive two doses of vaccine (1/5 the volume of the bovine dose) with a 21-days interval and are sampled at 0 and 30 dpv. The two dose regimen chosen in the lab animal model allow detecting the immune response induced by vaccines of low potency. The 21 interval between doses was adopted in order to obtain a curve of Ab kinetic response similar to that obtained in bovines, but in a shorter period of time providing a faster alternative method for vaccine potency testing than the one conducted in bovines.

Ab titers determined by ELISA as higher than 3.02 in guinea pigs and 2.72 in bovines were associated to very satisfactory potency vaccines. Vaccines inducing Ab titers between 3.02 – 1.93 in guinea pigs and 2.72 – 1.69 in bovines resulted satisfactory (18). Whereas, vaccines which induced Ab titers lower than 1.93 in guinea pigs and 1.69 in bovines were considered non satisfactory for commercialization.

SPECIES	VACCINES POTENCY viral neutralization (VN)		
	NON SATISFACTORY	SATISFACTORY	VERY SATISFACTORY
GUINEA PIG	$\bar{y} < 1.31$	$1.31 \leq \bar{y} < 2.05$	$2.05 \leq \bar{y}$
BOVINE	$\bar{Y} < 1.27$	$1.27 \leq \bar{Y} < 1.96$	$1.96 \leq \bar{Y}$

Table 2. Cut offs determined by VN expressed as Ab neutralizing titers calculated by the Reed and Muench method. Mean Ab titer of groups of 5 guinea pigs, evaluated 30 days post vaccination (dpv) and groups of 5 seronegative bovines evaluated 60 dpv. Bovines receive two doses of vaccine with a 30-day interval, and are sampled at 0 and 60 dpv. Guinea pigs receive two doses of vaccine (1/5 the volume of the bovine dose) with a 21-days interval and are sampled at 0 and 30 dpv.

Neutralizing Ab titers higher than 2.05 in guinea pigs and 1.96 in bovines were associated to very satisfactory potency vaccines. Vaccines inducing Ab titers between 2.05 – 1.31 in guinea pigs and 1.96 – 1.27 in bovines resulted satisfactory. Whereas, vaccines which induced Ab titers lower than 1.31 in guinea pigs and 1.27 in bovines were considered non satisfactory and therefore unsuitable for commercialization.

Either by ELISA or VN, vaccines classified as very satisfactory or satisfactory comply with the requirements established by the American 9.CFR, USA and the OIE Manual of diagnostic tests and vaccines of terrestrial animals for approval. Regarding protection against infection, a reduction of 1/100 or higher of the titer of infectious virus shed by vaccinated animals as compared to the titer shed by unvaccinated controls is requested. In the challenge assay performed with representative vaccines of the very satisfactory and satisfactory categories, in animals vaccinated with both vaccines, the amount of virus shed is significantly reduced when compared to control. Furthermore, virus shed by animals vaccinated with a very satisfactory vaccine was significantly lower than the one shed by the group receiving the satisfactory vaccine. In relation to the duration of clinical signs, the OIE demands a reduction of at least three or more days, with respect to the duration of the disease in controls. This requirement was only fulfilled by the very satisfactory vaccine. However, when more appropriate measurements are used to assess the disease, such as the area under the curve which considers severity and duration of clinical symptoms, both vaccine categories significantly reduce the signs of the disease.

Following this criterion, in order to evaluate agreement between bovines and the guinea pig model, 63 parallel trials were carried out in both species which included the calibration vaccines used in the dose-response assay, groups inoculated with placebo, unvaccinated groups and 22 commercial vaccines of unknown quality. Concordance was estimated by the kappa coefficient and results were (K)=0.894; ASE = 0.041; 95% CI 0.813–0.974; $p < 0.0001$, for Ab determined by ELISA and K=0.876, ASE = 0.050; 95%CI 0.777–0.971; $p < 0.0001$, for

neutralizing Ab. This indicates a very good agreement between the potency estimated for the guinea pig model and the one obtained in the target species (19).

The guinea pig model succeeded in adequately predicting not only vaccines immunogenicity, but also the efficacy grade when experimentally challenged in bovines. The proposed test does not need complex technology nor infrastructure, just an animal facility with guinea pigs and common serological techniques (ELISA, VN) of routine use in virology laboratories, appropriately harmonized with international norms (9CFR, OIE, EMEA) and preferably validated following norms ISO-IEC 17025 (20, 21, 22, 23).

Validation criterion for guinea pig testing

Potency testing in guinea pigs is considered valid when the mean Ab titer obtained from animals vaccinated with a reference vaccine results to be the expected value (24), and unvaccinated control animals (controls) remain seronegative for Ab against BoHV-1 throughout the experience.

Vaccine approval criterion by potency testing in guinea pigs. a) ELISA

All serums of animals immunized with a control vaccine will be evaluated. FIVE (5) serums with the highest titers obtained will be selected and an average will be calculated on that basis. For the **APPROVAL** of the vaccine submitted to control, mean Ab titers at 30 dpv must be higher or the same as **1.93** for ELISA technique for BoHV-1.

b) VIRAL NEUTRALIZATION

All serums of guinea pigs immunized with the vaccine submitted to control will be evaluated. FIVE (5) serums with the highest titers obtained will be selected and an average will be calculated on that basis. For **APPROVAL** of the vaccine submitted to control, mean Ab titers at 30 dpv must be higher or the same as **1.31** for VN technique for BoHV-1.

Harmonization of assays for the region

A positive and negative control serum panel and reference vaccines will be elaborated and made available for regional users to harmonize the results obtained for each assay laboratory adopting the control method. Local reference serums (from guinea pigs and bovines) will be traceable in the described techniques to European reference bovine serums (EU1, EU2 y EU3) provided by OIE Reference Laboratories.

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ANEX I

POTENCY TESTING FOR IBR IN GUINEA PIGS**Guinea pig conditions.**Animals must be more than 30 days old and weight 400 grams \pm 50 grams.For each batch under control, at least SIX (6) GUINEA PIGS will be vaccinated. Males and females may be used but each group must contain animals of the same sex.

Animal quarantine.Animals will have an adaptation period of SEVEN (7) days, at least, after entering the inoculation room.

Vaccine inoculation.A vaccine of 1/5 the volume of the bovine dose is applied subcutaneously.

Blood extraction to obtain serum samples. It can be collected by cardiac puncture, jugular vein or auricular vein, without anticoagulant. Samples are clarified by centrifugation, fractioned in 500 ul aliquots and stored at -20°C until analysis. Sample identification: label with date, protocol number, vaccine ID, guinea pig number, DPV time.

SEROLOGICAL CONTROLS FOR POTENCY TEST IN GUINEA PIGS**ELISA ASSAY** **FOR THE DETECTION OF ANTIBODIES AGAINST IBR**

A validated indirect ELISA is used to detect antibodies anti-BoHV-1 (20). Briefly, plates are sensitized with BoHV-1 virus, obtained from infected MDBK cells (positive well) or MDBK cells as negative control of infection (negative well). Optical density of virus and uninfected cells to sensitize plates is determined by crossed titration for each batch produced and it is constant for all the plate.Serums are assayed in both wells (+ and -) in 6 serial 4 fold dilutions starting from a minimum dilution of 1/40. The assay is developed using an Ab anti IgG (H+L) from guinea pig marked with peroxide as detection antibody. H₂O₂/ABTS is used as chromogen substrate system and reading is performed by an ELISA reader at 405 nm.

REAGENTS

- **Plates sensitization buffer (carbonate/bicarbonate) pH 9.6.**

Na₂CO₃ 0.159 g.

NaHCO₃ 0.293 g.Distilled water q.s.100 ml.

Adjust pH with NaOH/ HCl 1 NStore at 4° C (1-8° C).

- **Citric Acid Buffer pH 5.0**

Citric acid monohydrate 0.960 grs.

NaOH1N aprox.10 ml to reach pH 5.0

Distilled water q.s. 100 ml.

Adjust pH to:5.0 \pm 0.5 with Na(OH) or HCl 1N.Store at 4° C (1-8° C).

- **ABTS mother solution**

ABTS 0.22 g.

Citric acid buffer 10 ml.

Aliquote 1 ml. in plastic tubes.

Store at -20 \pm 5° C.

- **Revealing solution ABTS**

ABTS mother solution 300 ul.

Citric acid buffer pH5 10 ml.

Hydrogenperoxide 30 volume (H₂O₂) 10 ul.

- **Stop solution: SDS** (sodium dodecyl sulfate/ sodium laurilsulfate) in 5% water.Store at room temperature.

- **Wash buffer** (PBS, pH 7.4- Tween₂₀ 0.05%)

PBS pH 7.4 1000 ml.

Tween₂₀ 500 μ L.

- **Blocking Buffer and diluent.**PBS/Tween20 0.05%/OVA 1%, pH 7.4

Tween₂₀ 500 ul.

PBS 1X 1000 ml.

Ovalbumin 10 g.

Aliquote 50 ml in plastic tubes.Store at $-20 \pm 5^\circ$ C.

REAGENTS FOR SENSITIZATION

Positive capture control – Antigen: Preparation based on MDBK cell cultures infected with BoHV-1 reference strain.

Negative capture control:Preparation based on MDBK cell cultures.

Conjugated detection antibody Anti-IgG conjugate marked with peroxidase. The following can be used:Affinity purified goat anti- Guinea Pig Ig G (H+L) peroxidase labeled, KPL, cat.# 14-17-06.Peroxidase- conjugated affiniPure Goat anti-guinea pig IgG (H+L), Jackson, cat. # 106-035-003Note:Conjugates from other suppliers or produced in-house previously verified/ validated as optimal for ELISA assay can be used after corresponding titration with reference serums.

CONTROLS

Guinea pig positive control: Serum pool of 5 guinea pigs vaccinated with two doses of vaccine containing 10^7 DICT₅₀/ml of BoHV-1 in oil adjuvant (Reference Vaccine). Assays will be accepted when positive controls fall within the mean value ± 1 standard deviation (SD).

Mean value of corrected absorbance ± 1 SD = 0.520 \leq 0.740 \leq 0.960

The given serum, analyzed by seroneutralization must show an anti BoHV-1 neutralizing antibodies titer between 2.4 and 3.0 (Titer expressed using the Reed and Muench method). **Guinea pig negative control:** Guinea pig serum whose corrected absorbance in the dilution used results minor to technique cut off (40% corrected absorbance of positive control).

Reagent blank:PBS.For each control, 4 wells are used (two positive captures and its two corresponding negative captures)Also, it is advisable to include in each assay a positive sample of known titer and another negative sample

(internal standard serums). These samples are randomly set in different places in at least two assay plates and run in all assays.

ELISA PROCEDURE:

Plates coating

2. Perform the dilution used for the antigen and its negative capture control in coating buffer, pH 9.6. Use 96-wells ELISA plates "immulon 1b type". Place 50 µl of antigen in rows B-D-F-H and 50 µl of negative capture in rows A-C-E-G. Incubate during 17 hours ±2 h., between 4° C and 8° C.
3. Discard the content of the plate. Wash 3 times with wash buffer (PBS/Tween₂₀ 0.05 %, pH 7.4)
4. Add 100 µl/well of blocking buffer (PBS Tween₂₀ 0.05%/OVA 10%, pH 7.4). Incubate in humid chamber for 1 hour, at 37° C.
5. Next, discard blocking buffer, wash 3 times and either proceed with the assay or save the plate at -20°C, for 30 days maximum.

Dilution and setting of samples For the dilution of samples, the use of 96-wells culture plates is recommended. Place 195 µl of blocking buffer in column 1 and 7 and 150 µl in the remaining columns. Add 5 µl of serums to be analyzed and place them in pairs of wells 1 AB, 1 CD, 1 EF, 1 GH, 7 CD, 7 EF, 7 Gh (initial serum dilution 1/40). 7 samples fit per plate. Wells 7-12 AB are to assay controls. Carry out 4 fold dilutions transferring 50 µl from 1-6; discard tips. Using new tips carry out dilutions of 7-12.

9. Transfer 50 µl of each dilution performed to the reaction plate starting with the most diluted dilution to the most concentrated one.

Dilution and setting of controls

10. ELISA kit will be provided with standardized controls prepared in the following way: Place 200 µl of diluent (PBS Tween₂₀ Ova 10% pH 7.4) in two tubes and 4 µl of positive control serum in one of the tubes and 4 µl of negative control serum in the other. Homogenize.
11. Add 50 µl of the positive control dilution in wells A7 B7 A8 and B8 and 50 µl of negative control dilution in wells A9 B9 A10 and B10 and add 50 µl of PBS Tween₂₀ Ova 10% pH 7.4 in wells A11 B11 A12 and B12 (Reagent blank). Incubate in humid chamber during 1 h. at 37° C .
12. Discard content of the plate. Wash 4 times. Dry.
13. In a tube containing 5000 µl of diluent add the corresponding quantity of conjugated antibody following the dilution used. Add 50 µl per well in all the plate. Incubate in humid chamber during 1 hour, at 37° C.
14. Discard the content of the plate. Wash 5 times. Dry.

Development, Reading and Interpretation

15. Prepare 5 ml of developing solution. Add 50 µl of developing solution in each well and wait between 10 and 15 minutes with the plate in darkness. Read the assay at 405 nm to control that the positive control reaches the optical density expected in the established time range.
16. Stop reaction adding 50 µl of stop solution (SDS 5%) in all plate wells and read. Transfer reading data to a calculationsheet.

17. Subtract each absorbance of the negative captures from their respective positive captures.(Example:H1 minus G1= ODc (Corrected optical densities).
18. Calculate the mean of ODc of the positive control (100% PP).
19. Calculate the PP% of each sample in each dilution [PP = (ODc sample / ODc Positive Control)*100]
20. Calculate the mean of the replicates of the negative control and its PP%.

ASSAY ACCEPTANCE (CONFORMITY)

The following described criteria will be applied individually to each plate.

- An assay plate is accepted when ODc of the positive control is within the established range: 0.520 - 0.960. Negative control and reagents blank show PP% lower to assay cut-off (40%PP). Positive reference serum titer results in the expected value +/- a 4 fold dilution (error of the method).
- Antibodies titer of a sample is established as the reciprocal of the maximum dilution, whose PP% is higher or the same as the assay cut-off (40%PP).

RESULTS REPORT OF IMMUNOGENIC QUALITY OF IBR VACCINES TESTED IN GUINEA PIGS AND SERUMS EVALUATED BY ELISA.

Results can be interpreted and used to classify a vaccine in the guinea pig model by ELISA only if the ELISA assay has been accepted and you count with a minimum of 5 animals with results to estimate the mean Ab titer induced by the vaccine.

To validate the assay, serums of guinea pigs immunized with the reference vaccine must result in a mean titer within the established range determined by a control chart which shows the mean value \pm 2 standard deviations obtained from a minimum of 5 tests.

For the vaccine under evaluation, the mean antibody titer anti-BoHV-1 detected by ELISA as the average of the titers of 5 animals is informed (log10 of the reciprocal of the maximum dilution whose percentage of positivity is higher or the same as the assay cut off, established as higher or the same as 40% of the positive control). Negative samples in the minimum serum dilution assayed (1/40) are expressed as an arbitrary titer of 0.3 for calculation purposes.

VIRAL NEUTRALIZATION ASSAY TO DETERMINE AB FOR IBR

REAGENTS

Virus used:BoHV-1 Los Angeles (LA) reference strain or native BoHV-1 virus strain.Diluted so as to contain 100 tissue culture infectious dose 50% (DICT₅₀).

Controls: Guinea pig positive control: Serum pool of 5 guinea pigs vaccinated with two doses of vaccine formulated with 10⁷ DICT₅₀/ml of BoHV-1 in oil adjuvant (Reference Vaccine) with a neutralizing Ab titer of 2.4-3.0.

Guinea pig negative control: Normal guinea pig serum pool (pre-immunized guinea pig serums or unvaccinated controls).

Positive standard: Immunized guinea pig serum, with known VN Ab titer. **Negative standard:** Normal guinea pig serum.

Cell suspension: A cell suspension of MDBK line containing 200.000-250.000 cel/ml is used.

Samples inactivation: Before being used in a VN assay, serum samples, including assay controls, must be heat at water bath at $56 \pm 3^\circ \text{C}$, during 30 ± 5 minutes, to inactivate the complement.

Preparation of working medium: MEM-E supplemented with 1% antibiotic solution (0.5 gentamicin sulfate, 0.7% streptomycin sulfate, 0.2% penicillin G sodium) and 2% of bovine fetal serum (BFS).

VIRAL NEUTRALIZATION ASSAY PROCEDURE WITH FIXED VIRUS- VARIABLE SERUM:

1. 96-wells culture plates are used. Place 75 μ l/well of medium in all plates to be used.
2. **Design of plate for serums tested:** Add 25 μ l of the sample being tested, in quadruplicate. Start with a minimum dilution of 1/4 which summed to the volume of virus and cells results in an initial dilution of 1/8. Include standards of known titer at random among the samples to be analyzed.
3. **Design of control plate:** Positive and negative control serums are placed in the same way as the samples. To perform the cell control, add 150 μ l of working medium per quadruplicate in four rows (16 wells in total). For the control of the 100 DICT₅₀ of virus, three 10 fold dilutions are carried out based on the work dilution. 75 μ l of the 4 folded dilutions prepared are set in quadruplicate (Pure, 1/10; 1/100 and 1/1000) and 75 μ l of medium is added.
4. Carry out 4 fold serial dilutions, transferring 25 μ l, for all samples and control serums.
5. A toxicity control is carried out for each sample, adding 75 μ l of medium in another plate.
6. Prepare the dilution of the work virus (100 DICT₅₀) in the work medium. Add 75 μ l of the dilution of work virus in all plates, except for the plate of toxicity controls, in the cells control and in the 100 DICT₅₀ control.
7. Carry out three 10 fold dilutions of the work virus (pure, 1/10; 1/100; 1/1000) set 4 replicates of each dilution in a separate plate.
Incubate the plates (serum-virus blend) during 1 hour at 37°C in an atmosphere containing 5% CO₂.
8. Add 100 μ l of the cell suspension containing 200.000-250.000 cel./ml per well to the serum-virus blend, in all plates. Incubate plates at $37 \pm 1^\circ \text{C}$ in an atmosphere containing $5 \pm 1\%$ CO₂ during 48-72 h.

Reading and interpretation

After 48-72 h., reading is performed by inspection of monolayers in an optical microscope. Reading is by observation of viral cytopathic effect (CPE) typical of bovine herpes virus. Wells presenting a CPE typical of BoHV-1 are considered positive. In toxicity controls, monolayer must be observed the same as the cells controls, free of CPE and free of toxic effect. The neutralizing titer of the analyzed serum is obtained by the quantity of protected replicates in the serial dilutions based on the Reed and Muench interpolation method. If a certain serum presents toxicity in the analyzed dilutions, the neutralizing antibodies titer won't be determined by this technique.

ASSAY ACCEPTANCE (CONFORMITY)

The assay is accepted when:

- Monolayers of cell controls are in good conditions (confluent monolayers, light-refracting cells, with no morphological alterations, with no signs of contamination and with no BoHV-1 CPE).
- Viral suspension titer contains 100 DICT₅₀, with an admitted range of 50-200 DICT₅₀.
- Positive control shows the expected titer ± 1 well.
- Negative control results negative. An arbitrary value 0.3 is assigned for calculation purposes.

REPORT OF RESULTS OF IMMUNOGENETIC QUALITY OF IBR VACCINES TRIED/ TESTED IN GUINEA PIGS AND SERUMS EVALUATED BY VN

Results can be interpreted and used to classify a vaccine in the guinea pig model by VN only if the viral neutralization assay has been accepted and you count with a minimum of 5 animals with results to obtain the mean Ab titer induced by the vaccine. To validate the assay, serums of guinea pigs immunized with the reference vaccine must show a mean titer within the range established by a control chart which shows the mean value \pm two standard deviations obtained from a minimum of 5 samples.

For the vaccine under evaluation, mean neutralizing Ab titer anti-BoHV-1 obtained by the Reed and Muench method of the 5 immunized guinea pigs must be informed. Negative samples in the minimum serum dilution assayed (1/8) are expressed as an arbitrary titer of 0.3 for calculation purposes.