$$Val = \frac{\text{Delta OD spl} * 100}{\text{Delta OD pos}}$$

Using the first table in the quality control procedure, determine each sample’s status (positive, negative).

Distribuito in ITALIA da
Li StarFish S.r.l.
Via Cavour, 35
20063 Cernusco S/N (MI)
telefono 02-92150794
fax 02-92157285
info@listarfish.it
www.listarfish.it

BIO-X RESPIRATORY PULMOTEST (BIO K 340)

(Sandwich ELISA test for BoHV-1, BVDV, BRSV and Parainfluenza 3 detection)

KIT FOR ANTIGENIC DIAGNOSIS OF BOVINE RESPIRATORY VIRUSES BY ELISA

I - INTRODUCTION

Respiratory disorders are of major concern for bovidae, given the frequency of such infections and the high number of animals affected. These infections occur in all countries that practice intensive livestock farming in which large numbers of animals are confined to small areas. Treatment and diagnosis are both complicated due to the multifactorial character of this diseases etiology. Viruses and bacteria combined with stress due either to transport in overcrowded vans or dirty or poorly-ventilated stabling, for instance, play a key role in triggering acute respiratory infections. These infections are particularly common among young animals, although they also affect adult animals.
In most cases the animals that show signs of respiratory ailments harbour several pathogens, some of which may act synergistically. So, it is generally recognised that viruses are the first pathogens to intervene, whereas bacteria act as second invaders to worsen the animal's condition. Shipping fever is a good example of the synergism that can exist between a virus (PI3) and a bacterium, such as *Manheimia haemolytica*, in the respiratory tract.
Respiratory Pulmotest can be used to obtain a diagnosis from a minced lung tissue sample taken from a corps.

II - PRINCIPLE OF THE TEST

Specific antibodies produced against pathogens responsible of respiratory diseases have been immobilized on alternate rows of 12 x 8-well microtitre plates. These antibodies allow the specific capture of the corresponding pathogens in the samples. Rows A, C, E, G have been sensitised with these antibodies and rows B, D, F, H contain non-specific antibodies. These rows allow the differentiation between specific immunological reactions and non-specific binding so as to eliminate false positives.
The samples are diluted in lysis solution and incubated on the microplate for 1 hour at 21°C +/- 3°C.
After this first incubation step, the plate is washed and incubated for 1 hour with conjugates, peroxidase labelled anti-virus specific monoclonal antibodies. After this second incubation, the plate is washed again and the chromogen (tetramethylbenzidine) is added. This chromogen has the advantages of being more sensitive than the other peroxidase chromogens and not being carcinogenic.
If specific pathogens are present in the tested samples, conjugates remain bound to the corresponding microwells and the enzyme catalyses the transformation of the colourless chromogen into a pigmented compound. The intensity of the resulting blue colour is proportionate to the titres of the specific pathogens in the sample. The enzymatic reaction can be stopped by acidification and the resulting optical density at 450 nm can be recorded using a photometer. The signals recorded for the negative control microwells are subtracted from the corresponding positive microwells optical densities.

Control antigens are provided with the kit so as to validate the test results. These control antigens are composed of inactivated and lyophilised virus cultures.

III – COMPOSITION OF THE KIT

- **Microplates** : two 96-well microtitration plates. The rows A, C, E, G are sensitised by specific antibodies, the rows B, D, F, H by non specific antibodies.

Row A :	anti-BoHV-1	Row B :	control
Row C :	anti-BVDV	Row D :	control
Row E :	anti-BRSV	Row F :	control
Row G :	anti-Parainfluenza 3	Row H :	control

- **Washing solution** : One 100-ml bottle of 20x concentrated washing solution. The solution crystallises spontaneously when cold. If only part of the solution is to be used, bring the bottle to 21°C +/- 3°C until disappearance of all crystals. Mix the solution well and remove the necessary volume. Dilute the buffer 1:20 with distilled or demineralised water. Store the diluted solution at 4°C.
- **Lysis buffer** : One 50-ml bottle of 5x concentrated buffer. Dilute this concentrated solution 1:5 with distilled or demineralised water. Store the diluted solution at 4°C.
- **Conjugates** : - Four 6 ml vials of coloured conjugate. **These solutions are ready to use.** BHV-1 (red), BVDV (yellow), BRSV (blue) et Parainfluenza 3 (green). Specificity of conjugates is indicated on vials.
- **Control antigens :**
  - 2 vials containing the BoHV-1 control antigen (red cap).
  - 2 vials containing the BVDV control antigen (yellow cap).
  - 2 vials containing the BRSV control antigen (black cap).
  - 2 vials containing the PI3 antigen (green cap).

  Reconstitute these antigens with 1 ml of distilled or demineralised water. The reconstituted antigens may be kept at -20°C. Divide the reconstituted antigens into four portions before freezing in order to avoid repeated freezing and thawing. If these precautions are taken the reagent may be kept for several months.
- **Single component TMB** One 25-ml bottle of the chromogen tetramethylbenzidine (TMB). Store at 4°C protected from light. **This solution is ready to use.**
- **Stopping solution :** One 15-ml bottle of the 1 *M* phosphoric acid stop solution.

IV - PRECAUTIONS FOR USE

- This test may be used for *in vitro* diagnosis only. It is strictly for veterinary use.
- The reagents must be kept at between 4 and 8°C. The control antigen must be kept at -20°C once reconstituted. The reagents cannot be guaranteed if the shelf-life dates have expired or if they have not been kept under the conditions described in this insert.
- Do not use reagents from other kits.
- The quality of the water used to prepare the various solutions is of the utmost importance. Do not use water that may contain oxidants (e.g., sodium hypochlorite) or heavy metal salts, as these substances can react with the chromogen.
- Discard all solutions contaminated with bacteria or fungi.
- The stop solution contains 1 M phosphoric acid. Handle it carefully.

V – PROCEDURE

A. SAMPLE PREPARATION

1. **Homogenised tissue**. Collect an approximately 1-gramme sample of lung tissue. Take this sample from areas in the apical lobes that show gross lesions. Place the lung tissue fragment in a Petri dish with 2 ml of lysis solution and snip it into tiny pieces with a pair of scissors. Homogenize, transfer the dish's contents to a test tube, and centrifuge at 500 g for 10 minutes to separate out the insoluble fragments on the bottom of the tube. Collect the supernatant for the ELISA test.
2. **Cell culture.** Respiratory Pulmotest may be used to test for viral growth in likely host cells (primary lines, VERO, MDBK and HEP2). In this case you may deposit the culture medium directly on the microplate.

B. ELISA

1- All components must be brought to 21°C +/- 3°C at least 30 minutes before use.
2- Remove the microplate from its wrapper.
3- Add 100-µl aliquots of the diluted samples to the wells as follows : sample 1 in wells of column 2, sample 2 in wells of column 3, etc. Do not forget positive controls : BoHV-1 control in wells A1 and B1, BVDV control in wells C1 and D1, BRSV control in wells E1 and F1, PI3 control in wells G1 and H1.
4- Incubate the plate at 21°C +/- 3°C for 1 hour. Cover the plate with a lid.
5- Rinse the plate with the washing solution prepared as instructed in the 'Composition of the Kit' section. To do this, empty the microplate of its contents by flipping it over suddenly over a sink. Tap the upside-down microplate against a sheet of clean absorbent paper so as to remove all liquid. Fill the used wells with a washing solution either by means of a spray bottle or by plunging the microplate in a suitably-dimensioned container, then empty the plate once again by flipping it over a sink. Repeat the entire operation twice, taking special care to avoid the formation of bubbles in the wells. Upon completing these three washes, go on to the next step.
6- Add 100 µl of the conjugate solutions per well. Incubate at 21°C +/- 3°C for 1 hour. Cover the plate with a lid.

Anti - BoHV-1 conjugate (red)	rows A and B
Anti - BVDV conjugate (yellow)	rows C and D
Anti - BRSV conjugate (blue)	rows E and F
Anti – PI3 conjugate (green)	rows G and H

7- Wash the plate as described in Step 5.
8- Add 100 µl of the chromogen solution to each well on the plate. The chromogen solution must be absolutely colourless when it is pipetted into the wells. If a blue colour is visible, this means that the solution in the pipette has been contaminated. Incubate at 21°C +/- 3°C and away from light for 10 minutes. Do not cover. This time interval is given as a guideline only, for in some circumstances it may be useful to lengthen or shorten the incubation time.
9- Add 50 µl of stop solution to each well.
10- Read the optical densities by means of a microplate spectrophotometer with a 450 nm filter. The results must be read as quickly as possible after the stop solution has been applied, for in the case of a strong signal the chromogen can crystallise and lead to incorrect measurements

VI – INTERPRETING THE RESULTS

Subtract from each reading in the odd rows (A, C, E, and G) the signal of the corresponding negative control well (B, D, F, and H) and write down the result (calculation of the difference in optical density, delta OD). To calculate this value allow for the possible existence of negative values. Proceed in the same way for the positive controls. The test can be validated only if the positive references give 10 minute optical density differences that are greater than the values for the quality control appended to the package insert.

Divide each resulting value by the corresponding value obtained for the corresponding positive control and multiply this result by 100 to express it as a percentage.