100 µl per microwell. At the time of distribution of the chomogen-substrate mixture on the plates the solution must be completely colourless. If a blue colour appears at this stage, this solution must be discarded and a new one made up using absolutely clean glassware and equipment.

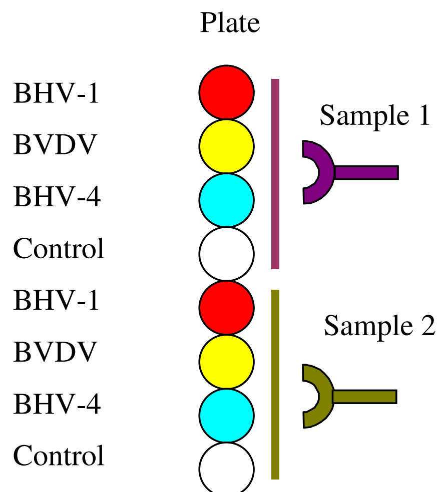
10- Incubate for 10 minutes at room temperature. This time is given as a guideline only, for in some circumstances it may be useful to lengthen or shorten the incubation time.
11- Add 50 µl of stop solution per microwell.
12- Read the optical densities in the microwells using a plate reader and a 450 nm filter. Results must be read fairly soon after the stopping solution has been added since the chromogen may cristallize in wells with strong signals and thereby distort the data.

VI – INTERPRETING THE RESULTS

Subtract from each value recorded in rows A, B, C the signal of the corresponding negative control (row D) and for the rows E, F, G the signal from the row H. Write down the result. Allow for any negative values that may exist in performing this calculation Carry out the same operations for the column corresponding to the positive control (wells A1 to D1).
The test can be validated only if the positive serum yields a difference in optical density at 10 minutes that is greater for each valence than the values given on the QC data sheet:

Divide the signal read for each sample well by the corresponding positive control serum signal and multiply this result by 100 to express it as a percentage. Using the first table in the quality control procedure, determine each serum's degree of positivity.

A reliable diagnosis can be made only if frank seroconversion can be documented using two coupled serum samples taken at 2- to 3-week intervals. The first sample must be taken during the acute phase of the infection. A frank seroconversion is considered to have occurred if the signal increases by two orders of magnitude (two plusses; for example, ++ -> ++++ or + -> +++). A sample must be considered positive if it yields a result that is greater than or equal to one plus sign (+).



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BIO-X ABORTION ELISA KIT (BIO K 072)

(Indirect test for blood sera)

KIT FOR SERODIAGNOSIS OF IBR/IPV, BVDV, BHV-4 INFECTIONS

IN CATTLE BY ELISA

I - INTRODUCTION

The task of determining the cause of an abortion in cattle is generally a rather difficult one because, most of the time, it is the consequence of an event which happened weeks to months earlier. Often also, the foetus is maintained in the uterus for hours and even days after its death, and, when it is finally evacuated, it has undergone autolysis, in such a way that it is difficult to do any type of analysis. Also, many causes of abortion in cattle are to this day still unknown. Moreover, many pathogens are rarely looked for because they are difficult or dangerous to handle (*Coxiella burnetii, Chlamydia psittaci* ..). Pathogens directly or indirectly responsible for abortions are numerous and varied, which complicates the diagnosis. Amongst the major pathogens responsible for abortions, one can find viruses (IBR, BVDV, BHV-4), bacteria (*Brucella abortus, Corynebacterium pyogenes, colibacille, streptocoque, Coxiella burnetii, Leptospira hardjo, Ureaplasma diversum, Campilobacter foetus, Borrelia coriaceae, Yersinia pseudotuberculosis, Chlamydia psittaci, Salmonella, Listeria monocytogenes et Hemophilus somnus*), parasites (*Distoma hepatica, Tritrichomonas, Sarcocystis, Neospora-like*), fungi (*Aspergillus fumigatus, Mortierella wolfii,* as well as *Mucor, Absidia, Rhizopus*) and yeasts (Candida).
The Bio-X Abortion ELISA kit concerns the three viruses BVDV, BHV-4 and IBR. This kit aims at demonstrating the existence of a seroconversion toward the three viruses mentioned above, in adult cattle, that it is, in the animal who aborted, but especially in the other animals of the herd, ideally in 10% of the livestock or the cowshed. Indeed, when the abortion occurs, the serological titer of the cow has often reached its maximum and it is not possible to show a seroconversion. It is thus preferable to test the other animals of the herd in order to verify whether the suspected infection is still active. If many animals show a clear seroconversion toward one of the three pathogens of the kit, one can attribute the abortion to this pathogen.

II – PRINCIPLE OF THE TEST

The test uses 96-well microtitration plates sensitised by monoclonal antibodies specific to the three pathogens listed above. These antibodies are used to trap the pathogens as well as to purify them from lysates of the cells in which the viruses were grown. The distribution of these pathogens on the microtitration plate is as follows:

Rows A et E : IBR,
Rows B et F : BVDV,
Rows C et G : BHV-4,
Rows D et H : negative control.

Rows D and H contain a lysate of the bovine kidney cell line that was used as a substrate to propagate the viruses. We thus have a genuine negative control to differentiate the virus-specific antibodies from those directed against the antigenic determinants of the kidney cells used for their replication. Using such a control reduces the number of false positives considerably. The test sera are diluted in an appropriate buffer and incubated on the plate for one hour at room temperature. The plate is washed and the conjugate --a peroxidase-labelled anti-bovine IgG1 monoclonal antibody-- is added to the wells. The plate is reincubated at room temperature for 1 hour. After this second incubation, the preparation is washed and the enzyme substrate (hydrogen peroxide) and the chromogen tetramethylbenzidine (TMB) are added. This chromogen has the advantages of being more sensitive than the other peroxidase chromogens and not being carcinogenic. If specific immunoglobulins are present in the test sera the conjugate remains bound to the corresponding microwell 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 titre of specific antibody in the sample. The signals recorded for the negative control microwells are subtracted from the corresponding positive microwells. It is possible to quantify the reactivity of an unknown serum on a scale ranging from 0 to +++++.

III - COMPOSITION OF THE KIT

- **Microplates**: Two 96-well microtitration plates. The distribution of the different valencies on the microtitration plate is as follows :

Row A:	IBR
Row B:	BVDV
Row C:	BHV-4
Row D:	negative control
Row E:	IBR
Row F:	BVDV
Row G:	BHV-4
Row H:	negative 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 room temperature 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.
- **Dilution buffer**: One 50-ml bottle of 5x concentrated buffer for diluting the blood sera and conjugate. Dilute this concentrated dilution buffer 1:5 with distilled or demineralised water. This solution will keep at 4°C for at least 3 months. If a deposit forms at the bottom of the container filter the solution on Whatman filter paper.
- **Conjugate**: One bottle of anti-bovine immunoglobulin-peroxidase conjugate (horseradish peroxidase-labelled anti-bovine IgG1 monoclonal antibody).
- **Positive serum**: One bottle of positive serum. Reconstitute this serum with 0.5 ml of distilled or demineralised water. The reconstituted serum may be kept at -20°C. Divide the reconstituted serum into several portions before freezing in order to avoid repeated freezing and thawing. If these precautions are taken the reagent may be kept for several months.
- **Chromogen solution**: One 2-ml drop-dispenser bottle of the chromogen tetramethylbenzidine. Store at 4°C.
- **Substrate solution**: One 30-ml bottle of the hydrogen peroxide substrate solution. Store this reagent at 4°C.
- **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 positive serum must be kept at -20°C once it is reconstituted. The reagents cannot be guaranteed if the shelf-life dates have expired or 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 make up 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 carefully.

V – PROCEDURE

1- Bring all reagents at room temperature at least 30 minutes before starting the test.
2- Remove the microplate from its wrapper.
3- Transfer 1 ml/tube of the buffer for dilution, prepared as instructed in the "Composition of the Kit" section, to 5- or 10-ml hemolysis tubes. Add 10 µl of the serum samples to each of these tubes and shake briefly on a mechanical agitator. Proceed in the same manner for the positive serum.
4- Add 100-µl aliquots of the dilute samples (1:100) to the wells as follows: positive reference serum: wells A1 to D1, sample 1 wells E1 to H1, sample 2 wells A2 to D2, etc.
5- If a diagnosis based on seroconversion monitoring is required, incubate the plate at room temperature for one hour. If, in contrast, the test's sensitivity is more important (positive-negative test), it is preferable to incubate the microplate at 37°C for one hour or on a plate agitator at room temperature for one hour. Shaking or 'stirring' during incubation will improve the test's sensitivity. This will, however, yield signals for certain sera that are greater than 2 optical density units. Not all microplate readers can interpret such values.
6- Rinse the plate with the washing solution, prepared as instructed in the "Composition of the Kit" section, as follows: Empty the microtitration plate of its contents by flipping it over sharply above a sink. Tap the plate upside down against a piece of clean absorbent paper to remove all the liquid. Fill the utilised wells with the washing solution using a squeeze bottle or by plunging the plate in a vessel of the right dimensions, then empty the wells once more by turning the plate over above a sink. Repeat the entire operation two more times, taking care to avoid the formation of bubbles in the microwells. After the plate has been washed three times go on to the next step. An automatic plate washer may also be used, but in this case particular care must be taken to avoid any contact between the needles and the bottom of the wells to prevent any damage of the reagent layer.
7- Dilute the conjugate 1:50 in the buffer for dilution (for example, for one plate dilute 250 µl of the conjugate stock solution in 12.250 ml of diluent). Add 100 µl of the dilute conjugate solution to each well. Incubate for 1 hour at room temperature, at 37°C or on an agitator, depending on the choice made in Step 5.
8- Wash the plate as described in step 6 above.
9- Prepare 10 ml of indicator solution extemporaneously as follows: Add 12 drops (500µl) of chromogen to 9,5 ml of the substrate solution. Mix thoroughly, then apply to the plate immediately in volumes of