Bovine adenovirus 3

ELISA kit for serodiagnosis of Bovine Adenovirus 3
Indirect test for blood sera, plasma and milk
Diagnostic test for cattle
Double wells

I - INTRODUCTION

Bovine adenoviruses (BAV) are classified into nine serotypes (species) so far. These nine serotypes are divided into two subgroups mainly on the basis of replication in either calf kidney or testicle cells (subgroup I, serotypes 1-3 and 9) or only in testicle cells (subgroup II, serotypes 4-8). The two subgroups also differ in their antigenic properties. The distribution of BAV in cattle populations is worldwide. Serological evidence suggests a high incidence of infection. Adenovirus are generally present in large herds. Viruses are excreted by different forms of shedding and by coughing. Infection occurs most frequently when calves are 3 weeks to 4 months old. In diseased calves respiratory and enteric symptoms, fever and anorexia can be observed. The disease usually starts with respiratory symptoms. Serous excretions from the nose and conjonctivae are accompanied by coughing. Enteric infection becomes manifest by salivation and thin, greyish-yellow feces. The disease can be aggravated by secondary bacterial infections. For the diagnosis of the disease, paired serum samples should be taken from calves and tested by ELISA method. If no acute disease is observed but infection of the herd is suspected, a serological survey can be performed. In this case, single serum samples are collected from adult animals representing 5-10 % of the herd. Bio-X bovine adenovirus 3 ELISA kit is subgroup-specific and can only detect immune response against subgroup I bovine adenovirus.

II – PRINCIPLE OF THE TEST

The test uses 96-well microtitration plates sensitised by monoclonal antibodies specific to one of the antigenic determinants of adenovirus 3. This antibody is used to trap the virus as well as to purify it from lysate of the cells in which the virus was grown. The plate's odd columns (1, 3, 5, 7, 9 and 11) contain the virus, whereas the even columns (2, 4, 6, 8, 10 and 12) contain a lysate of bovine kidney cell line that was used as a substrate to propagate the virus. We thus have a genuine negative control to differentiate the specific anti-viral antibody from the antibodies directed against the antigenic determinants of the bovine kidney cells used for its replication. Using such a control reduces the number of false positives considerably.

The test blood sera, plasma or milks are diluted in the buffer for dilution. The plate is incubated and washed, then the conjugate, a peroxidase-labelled anti-bovine IgG1 monoclonal antibody, is added to the wells. The plate is then incubated a second time at 21°C +/- 3°C and 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 adenovirus 3 immunoglobulins are present in the sample the conjugate

remains bound to the microwell that contains the viral antigen and the enzyme catalyses the transformation of the colorless chromogen into a pigmented compound. The intensity of the resulting blue colour is proportionate to the titre of specific antibody in the sample. The signal read off the negative control microwell is subtracted from that of the positive microwell sensitised by the viral antigen. 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 odd columns (1, 3, 5, 7, 9 and 11) are sensitised by the adenovirus 3 viral antigen and the even columns (2, 4, 6, 8, 10 and 12) by the cells lysate.
- **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 so that all the crystals disappear. Mix the solution well and remove the necessary volume. Dilute the buffer 1:20 with distilled or demineralised water.
- **Dilution buffer**: One 50-ml bottle of 5x colored, concentrated buffer for diluting the blood sera, plasma, milks and conjugate. The bottle's content is to be diluted with distilled or demineralised water. If a deposit forms at the bottom of the receptacle filter the solution on Whatman filter paper.
- **Conjugate**: 1 bottle of anti-bovine immunoglobulin-peroxidase conjugate (horseradish peroxidase-labelled anti-bovine IgG1 monoclonal antibody).
- **Positive reference**: One bottle of positive serum. Store this reagent between +2°C and +8°C.
- **Negative reference**: One bottle of negative serum. Store this reagent between +2°C and +8°C.
- **Single component TMB**: One 25-ml bottle of the chromogen tetramethylbenzidine (TMB). Store between +2°C and + 8°C protected from the light.
- **Stop solution**: One 15-ml bottle of the 1 M phosphoric acid stop solution.

	BIO K 063/2
Microplates	2
Washing solution	1 X 100 ml (20 X)
Colored Dilution buffer	1 X 50 ml (5 X)
Conjugate	1 X 0.5 ml (50 X)
Positive serum	1 X 0.5 ml (1 X)
Negative serum	1 X 0.5 ml (1 X)
Single component TMB	1 X 25 ml (1 X)
Stop solution	1 X 15 ml (1 X)

IV - ADDITIONAL MATERIALS AND EQUIPMENT REQUIRED

Distilled water, graduated cylinders, beakers, plastic tubes, tube rack, microplates dilution, dispenser tips, reagent reservoir for multichannel pipettes, lid, adhesive for microplates, graduated automatic (mono- and multichannel) pipettes, microplate reader, and microplate washer and shaker (optional)

V - PRECAUTIONS FOR USE

- This test may be used for "in vitro" diagnosis only. It is strictly for veterinary use.
- The reagents must be kept between +2°C and +8°C. 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.
- The concentrated wash solution and dilution buffer may be stored at room temperature. Once diluted, these solutions remain stable for six weeks if kept between +2°C and +8°C.
- Unused strips must be stored immediately in the aluminium envelope, taking care to keep the desiccant dry and the envelope's seal airtight. If these precautions are taken, the strips' activity can be conserved up to the kit's shelf-life date.
- 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.

- All materials and disposable equipment that come in contact with the samples must be considered potentially infectious and be disposed of in compliance with the legislation in force in the country.
- To guarantee the reliability of the results, one must follow the protocol to the letter. Special care must be taken in observing the incubation times and temperatures, as well as measuring the volumes and dilutions accurately.

VI – PROCEDURE

1- Bring all components to 21°C +/- 3°C before use. Remove the microplate from its wrapper.

2- DILUTION OF SAMPLES

2.1- Blood sera and plasma preparation

The blood serum and plasma samples must be diluted 1:100. Avoid using haemolysed samples or those containing coagulum.

2.1.1- Dilution in tubes

Distribute 990 µl aliquots of dilution buffer, prepared as instructed in the section "Composition of the Kit", to 5 or 10 ml tubes. Add 10 µl aliquots of the samples to each of these tubes and mix briefly on a mechanical stirrer (final dilution: 1:100).

2.1.2- Dilution on a microplate

Distribute 20 µl aliquots of each of the samples to the microwells of a dilution plate. Add 180 µl of dilution buffer. Mix five times by pumping and surging or orbital agitation (dilution: 1:10). Distribute 90 µl aliquots of dilution buffer to the wells of the kit's microplate. Transfer 10 µl of the 1:10 prediluted samples. Mix five times by pumping and surging or orbital agitation (final dilution: 1:100).

2.2- Dilution of the kit's reference sera (positive and negative controls)

The positive and negative sera must be diluted 1:100. Do these dilutions in one step in a tube (see Point 2.1.1.) or in two steps on a dilution microplate (see Point 2.1.2.).

2.3-Milk preparation

Centrifuge at 4000 g for 20 minutes. Take up the middle layer of liquid by means of a glass Pasteur pipette inserted through the upper layer of cream, taking care not to touch the underlying cell sediment.
The milk samples must be diluted 1:4.

2.3.1- Dilution in tubes

Distribute 750 µl aliquots of dilution buffer, prepared as instructed in the section "Composition of the Kit", to 5 or 10 ml tubes. Add 250 µl aliquots of milk to each of these tubes and mix briefly on a mechanical stirrer (final dilution: 1:4).

2.3.2- Dilution on a microplate

Distribute 60 µl of each sample to the microwells of a dilution plate. Add 180 µl of dilution buffer. Mix five times by pumping and surging or orbital agitation (dilution: 1:4). Transfer 100 µl aliquots of the diluted samples to the kit's microplate.

3- Distribute the samples (blood serum, plasma, or milk) at the rate of 100 µl per well. For example, the following pattern may be followed: Positive serum in wells A1 and A2, Negative serum in wells B1 and B2, Sample 1 in wells C1 and C2, and so on. Cover with a lid and incubate the plate at 21°± 3°C for one hour.

4- Rinse the plate with the washing solution prepared as instructed in the section "Composition of the Kit". To do this, eliminate the microplate's contents by flipping it sharply over a container filled with an inactivating agent. Let the microplate drain upside-down on a sheet of clean absorbent paper so as to eliminate all liquid. Add 300 µl of the washing solution, and then empty the plate once again by flipping it over above the containment vessel. Repeat the entire operation two more times, taking particular care to avoid bubble formation in the wells. After these three rinses, go on to the next step.
Using a plate washer (whether automatic or manual) is also recommended. However, the depth of the needles' immersion must be set so as not to disturb the layer of reagents adsorbed to the bottom of each well.
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.

5- Dilute the conjugate 1:50 in the dilution buffer (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. Cover with a lid and incubate the plate at 21°± 3°C for one hour.
6- Wash the plate as described in step 4 above.
7- 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.
8- Incubate for 10 minutes at 21°C +/- 3°C protected from the light and uncovered. This time 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 per microwell. The blue colour will change into a yellow colour.
10- Read the optical densities in the microwells using a plate reader and a 450 nm filtre. 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.

VII – INTERPRETING THE RESULTS

Subtract from each value recorded for the odd columns the signal of the corresponding negative control well and write down the result. In performing this calculation, allow for any negative values that may exist. Carry out the same operations for the column corresponding to the positive control
The test can be **validated** only if the positive serum yields a difference in optical density at 10 minutes that is greater than 0,600 and the negative serum yields a difference in optical density that is lower than 0,200.

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.

$$\text{Val(ue)} = \frac{\text{Delta OD Sample} * 100}{\text{Delta OD positive}}$$

Using the following table, determine each serum's, plasma's or milk's degree of positivity.

0		+		++		+++		++++		+++++
Val <=	10 %	< Val <=	33 %	< Val <=	56 %	< Val <=	79 %	< Val <=	102 %	< Val

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 (+).**

VIII – ORDERING INFORMATION

Monoscreen AbELISA Bovine adenovirus 3 2x48 tests BIO K 063/2