Mycoplasma bovis

ELISA Kit for serodiagnosis of *Mycoplasma bovis*
Indirect test for blood sera, plasma and milk
Diagnostic test for cattle
Double wells

I - INTRODUCTION

Mycoplasma bovis is associated with many cattle diseases, including arthritis, pneumonia in calves and young stock, mastitis, and genital infections. The infectious pneumonias that affect intensively-raised calves are responsible for sizable economic losses due to the mortality, treatment costs, and growth delays that they cause. These respiratory infections often involve multiple factors and are caused by interactions among viruses, mycoplasmas, and bacteria. Several species of Mycoplasma have been isolated from the respiratory tracts of calves. Some of them are most probably simple commensals or opportunistic species that merely worsen the lung damage caused by other agents. *Mycoplasma bovis* has been isolated from the lungs of calves with pneumonia. It is probably the most pathogenic species affecting the Bovidae after *Mycoplasma mycoides mycoides. Mycoplasma bovis* can induce the development of pneumonia in gnotobiotic calves. *Mycoplasma bovis* is frequently found in association with *Mannheimia haemolytica* in pneumonia in calves.

II – PRINCIPLE OF THE TEST

The test uses 96-well microtitration plates sensitised by a recombinant protein from *Mycoplasma bovis* expressed by *E. coli*. A gene from *Mycoplasma bovis* is expressed by this recombinant *E.coli* culture. The plate's odd columns (1, 3, 5, 7, 9 and 11) contain the recombinant protein, whereas the even columns (2, 4, 6, 8, 10 and 12) contain a negative control antigen. We thus have a genuine negative control to differentiate the specific antibody. Using such a control reduces the number of false positives considerably.
The test blood sera and plasma are diluted in the dilution buffer. The milk samples are used undiluted. Samples are added to the plate which is then incubated and washed. The conjugate, protein G peroxidase-labelled, is added to the wells. The plate is incubated a second time at 21°C +/- 3°C. After the second incubation, the plate is washed again and the chromogen (tetramethylbenzidine) is added. This chromogen has the advantage of being more sensitive than the other peroxidase chromogens and not being carcinogenic. If specific anti-*Mycoplasma bovis* immunoglobulins are present in the test sera, plasma or milk the conjugate remains bound to the microwell that contains the bacterial recombinant 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 recombinant *M.bovis* protein. It is possible to quantify the reactivity of an unknown sample on a scale ranging from 0 to +++++.

III - COMPOSITION OF THE KIT

- **Microplates**: 96-well microtitration plates (6 strips of 16 wells). The odd columns (1, 3, 5, 7, 9 and 11) are sensitised by the recombinant *M. bovis* and the even columns (2, 4, 6, 8, 10 and 12) by the negative control protein.
- **Washing solution**: One 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 all crystals have disappeared. Mix the solution well and remove the necessary volume. Dilute the buffer 1:20 with distilled or demineralised water.
- **Dilution buffer**: One bottle of 5x concentrated buffer for diluting the blood sera, plasma and the 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**: One bottle of Protein G, horseradish peroxidase-labelled.
- **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.
- **Tracer**: One bottle of tracer. Store this reagent between +2°C and +8°C.
  The tracer is a reference sample that can be used to check the intra-laboratory reproducibility of the kit's batch.
  Intra-laboratory reproducibility: Degree of agreement between the results of reiterated tests on the same sample with an identical technical protocol in a given laboratory under variable working conditions.
- **Single component TMB:** One bottle of the chromogen tetramethylbenzidine (TMB). Store between +2°C and +8°C protected from light. **This solution is ready to use.**
- **Stop solution**: One bottle of the 1 M phosphoric acid stop solution.

	BIO K 260/2	BIO K 260/5
Microplates	2	5
Washing solution	1 X 100 ml (20 X)	1 x 250 ml (20 X)
Dilution buffer	1 X 30 ml (5 X)	1 x 100 ml (5 X)
Conjugate	1 X 0,5 ml (50 X)	1 X 1,4 ml (50 X)
Positive serum	1 X 0,5 ml (1 X)	1 X 0,5 ml (1 X)
Negative serum	1 X 0,5 ml (1 X)	1 X 0,5 ml (1 X)
Tracer	1 X 0,5 ml (1 X)	1 X 0,5 ml (1 X)
Single component TMB	1 X 25 ml (1 X)	1 x 55 ml (1 X)
Stop solution	1 X 15 ml (1 X)	1 x 30 ml (1 X)

IV - ADDITIONAL MATERIALS AND EQUIPMENT REQUIRED

Distilled water, graduated cylinders, beakers, plastic tubes, tube rack, microplates for 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 coagula.

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) and the tracer
The positive and negative sera and the tracer 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.
Use undiluted skimmed milk samples in the wells.

3- Distribute the samples (blood serum, plasma, or milk) using 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, Tracer in wells C1 and C2, Sample 1 in wells D1 and D2 and so on. Cover the plate with a lid and incubate 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, dispose of 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.
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 diluted conjugate solution to each well. Cover the plate with a lid and incubate for one hour at 21°C +/- 3°C.
6- Wash the plate as described 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 crystallize 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 and negative controls
The test can be **validated** only if the positive control serum yields a difference in optical density at 10 minutes that is greater than 0,800 and the negative control serum yields a difference in optical density that is lower than 0,300

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 <=	37 %	< Val <=	60 %	< Val <=	83 %	< Val <=	106 %	< Val <=	129 %	< 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 *Mycoplasma bovis*	2x48 tests	BIO K 260/2
	5x48 tests	BIOK 260/5