Mycoplasma bovis

Enzyme Immunoassay for the Detection
of Antibodies Directed Against Mycoplasma bovis

Instruction For Use

Order Codes: Kits for:
REF 712000 96 tests

Manufactured by:

MAST DIAGNOSTICA
Laboratoriums-Präparate GmbH
Feldstraße 20, D-23858 Reinfeld
Fon: +49 (0) 4533 2007-0
Fax: +49 (0) 4533 2007-68
E-Mail: mast@mast-diagnostica.com
http://www.mast-diagnostica.com
1. Introduction

*Mycoplasma* are intracellular bacteria. Characterised by the lack of a cell wall and reduced biochemical activity in their energy metabolism, they are found in many species like ruminants, buffalo and even humans. Infection with the species *Mycoplasma bovis* in cattle, however, is of substantial economical importance. *M.bovis* free herds are often infected by young asymptomatic animals which are brought into the herd. Once the infection has manifested, the eradication of *M.bovis* is not easily achieved. Infected animals shed *M.bovis* through the respiratory tract for many months, infecting animals in close proximity. Other infection pathways are the teat canal, the genital tract or infected semen used for artificial insemination. *M.bovis* positive frozen semen keeps its infectious potential for years.

Environmental infections are theoretically possible but are not considered to be the major cause. *M.bovis* can survive for more than 2 weeks in water, but with increasing temperatures the viability drops significantly. Direct animal contacts are the most frequent source of infection.

Disease symptoms vary from fever over nasal discharge, loss of appetite, hyperpnoea, dyspnoea to an increased mortality by severe serofibrinous pneumonia. As a consequence of these infections, highly contagious mastitis, arthritis and an increased abortion risk are found in *M.bovis* positive herds.

An antibody screening program for *Mycoplasma bovis* is highly recommended to monitor and control the infection and so minimise the economic losses. An infection can best be detected by ELISA. Since clinical signs are not clearly characteristic of an infection the detection of IgG antibodies is easy and reliable. The antibody titres remain elevated over a period of several months.

According to the OIE Manual of diagnostics and vaccines *Mycoplasma bovis* is a group 2 organism.

2. Principle of the Test

Diluted serum from cattle is incubated in microwells coated with *Mycoplasma bovis* antigen.

During incubation, any antibodies in the sample that are specific for *M.bovis* bind to the immobilised *M.bovis* antigen. The wells are washed to remove any unbound material and affinity purified rabbit anti-bovine immuno globulins conjugated with horseradish peroxidase, is added to the wells.

After a second incubation step, the wells are washed to remove any unbound material and the complexed enzyme is detected by the addition of chromogenic TMB substrate (3,3', 5, 5' tetramethylbenzidine). During incubation any complexed HRP enzyme will react with the substrate to produce a blueish colour. This reaction is stopped after a specific time with acid, which also causes a change in colour to an intense yellow.

The wells are then read spectrophotometrically. The intensity of the colour reaction is proportional to the amount of *M.bovis*-specific antibodies in the sample.
3. Contents

1. 12 x Microtiter strips with 8 pre-coated break-apart wells coated with Mycoplasma bovis native antigen; supplied in a strip holder, and sealed in an aluminium pouch with a sachet of silica gel.

2. 4 x Self-adhesive plate sealers.

3. 2 x 60 mL Sample diluent. Green coloured solution, supplied ready to use.

4. 1 x 12 mL Rabbit anti-bovine immuno globulins conjugated with horseradish peroxidase, diluted in a red coloured solution containing PBS buffer, horse serum and 0.1 % of Proclin as a preservative. Supplied ready to use.

5. 1 x 1.5 mL Positive control. Bovine serum with antibodies directed against Mycoplasma bovis. Supplied ready to use.

6. 1 x 1.5 mL Negative control. Supplied ready to use.

7. 1 x 50 mL Washing buffer, supplied 30x concentrated, consisting of:
   - 0.9 % (w/v) NaCl, final concentration
   - 0.05 % (w/v) Tween 20, final concentration
   - 0.003 % (w/v) Proclin, final concentration

8. 1 x 12 mL TMB substrate (3,3',5,5' tetramethylbenzidine), DMSO free reagent. Supplied ready to use.

9. 1 x 12 mL Stopping solution, contains 1 N H$_2$SO$_4$. Supplied ready to use.

4. Materials required but not provided

1. Vortex mixer.

2. Precision micropipettes both single and multichannel capable of dispensing 25 µL, 50 µL and 200 µL.

3. Disposable pipette tips.

4. A supply of distilled or deionised water (ultrapure or HPLC grade water).

5. A range of clean standard volumetric laboratory glass or plastic containers of 500 mL, 1000 mL or 2000 mL volume.

6. 37 °C incubator.

7. Manual washing system or an automatic positive pressure plate washer to fill and aspirate contents of wells. (N.B. plate washer relying on gravity fed washing solutions are not suitable.)

8. Absorbent paper towel.

5. **Warnings and Precautions**

1. The reagents supplied in this kit are for in vitro diagnostic use only
2. Read instructions carefully before conducting the assay. Do not modify the instructions procedure.
3. Do not use kit beyond the expiry date.
4. Do not interchange reagents between different kit lots as reagents have been calibrated for each kit.
5. Examine all kit reagents before performing an assay. Reagents should not be used if they appear cloudy or are suspect for any reason.
6. All reagents should be stored at 2 - 8 °C and brought to room temperature before use.
7. Do not re-use microwells.
8. Do not mouth pipette.
9. Use high quality distilled or deionised water (ultrapure or HPLC grade water) throughout.
10. Do not allow microwell to dry out during the assay procedure.
11. Do not heat-expose the chromogenic TMB substrate. TMB is flammable. In use avoid contact with skin, eyes and mucous membranes and keep away from heat and naked flames.
12. Protect solution from exposure to direct light. The substrate incubation step should be conducted in the dark.
13. The Stopping Solution contains H$_2$SO$_4$ which is corrosive. Avoid contact with skin, eyes and mucous membranes.
14. Use disposable plasticware where possible. Re-usable glassware should be washed thoroughly and rinsed free of detergents before use.
15. Do not cross-contaminate reagents or interchange caps on bottles. Use separate pipettes or pipette tips for each sample or reagent.
16. Do not cross-contaminate specimen between wells. If while dispensing, specimen or reagent is dropped on the surface of the well strips then blot dry immediately.
17. The local operating procedures for the containment of potentially infectious material have to be practised. Specimens may contain infectious organisms. Exercise extreme care at all times when handling specimen.
18. Dispose of all clinical and control material safely and in accordance with local operation regulations.

6. **Storage and Stability**

1. The MASTAZYME Mycoplasma bovis unused components can be used until the expiry date displayed on the label, if stored at 2 - 8 °C.
2. After opening store all reagents at 2 - 8 °C where possible. Once opened reagents are stable for at least 3 months, when not contaminated during use.

3. Working strength washing buffer may be stored in tightly capped containers at 15 - 30 °C for up to one month.

7. **Reagent preparation**
   Allow all reagents, microwells and test specimens to equilibrate to room temperature before proceeding further with the assay.

   Prepare a **1 in 30 dilution** of the concentrated washing buffer using ultrapure water as required or dilute the entire bottle (50 mL) with 1450 mL of ultrapure water.

   Dilute specimen (bovine serum or bovine plasma) 1:101 with sample diluent before testing. For example 10 µL (5 µL) of sample to 1000 µL (500 µL) of sample diluent in a suitable tube.
8. Assay Procedure

Make sure all reagents are at the bottom of the microtiter well. Carefully mix the reaction mix before incubation.

1. Prepare a sufficient number of microplate strips to accommodate the negative control, positive control and the diluted samples.

2. Pipette **100 µL of controls and samples** into the appropriate wells. **Cover** the **microwell strips with plate sealing film** provided to prevent evaporation of liquid from the wells. Incubate the plate for 30 min at 37 °C.

3. Discard the contents of the microwells and wash 4 times with 300 µL of washing solution. Blot dry the plates on absorbent paper.

4. **Dispense 100 µL of red coloured conjugate** into each well. Make a visual check to ensure all wells contain conjugate.

5. Incubate at **37 °C for 30 minutes**.

6. Discard the contents of the microwells and wash 4 times with 300 µL of washing solution. Blot dry the plates on absorbent paper.

7. **Dispense 100 µL of ready to use TMB solution** into all wells. A multichannel pipette is recommended for this step. Never use TMB solution if it has turned blue or has precipitated. **Cover the microwell strips with a fresh piece of plate sealing film.**

8. **Incubate** in the dark at room temperature for **5 ± 2 minutes**.

9. After incubation terminate the reaction by adding **50 µL of stopping solution** to all wells.

10. **Read results** after adding the stopping solution **within 30 min** using a suitable microplate reader with a **450 nm filter setting** (reference filter 620 - 690 nm). Blank the reader against air.
9. Calculation of Results and Interpretation

All OD values should be within the range of Certificate of Kit Performance which is supplied with each kit.

For calculation the value of the positive control measured with a 450 nm filter setting is set to 1.000 OD. All samples are referred to this value and re-calculated.

Example: Recalculated result = (1 / OD pos control) * OD sample

e.g. specimen 1:  
\[
\text{Recalculated result} = \left(\frac{1}{1.128}\right) \times 0.850 = 0.754
\]

<table>
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<th></th>
<th>OD 450 nm</th>
<th>Mean</th>
<th>Recalculated</th>
<th>Results</th>
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<tbody>
<tr>
<td>Positive Control</td>
<td>1.124</td>
<td>1.128</td>
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<tr>
<td></td>
<td>1.132</td>
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<tr>
<td>Negative Control</td>
<td>0.290</td>
<td>0.256</td>
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<td></td>
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<td>Specimen 1</td>
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<td>Specimen 6</td>
<td>0.206</td>
<td>0.183</td>
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</table>

10. Interpretation

Samples may fall into the following categories:

positive sample: mean OD (if tested in duplicate) > 0.300

negative sample: mean OD (if tested in duplicate) < 0.250

suspect sample: mean OD (if tested in duplicate) 0.250 – 0.299

11. Literature

(A) Nicholas RAJ, Ayling RD; Mycoplasma bovis: disease, diagnosis, and control; Res Vet Science, 74, 2003, 105-112

(B) Nicholas RAJ, Ayling RD, Stipkovitis LP; An experimental vaccine for calf pneumonia caused by Mycoplasma bovis: clinical, cultural, serological and pathological findings; Vaccine, 20, 2002, 3569-3575

(C) Pfutzner H, Sachse K; Mycoplasma bovis as an agent of mastitis, pneumonia, arthritis and genital disorders; Scientific and Technical Review, Offices International Des Epizooties, 15, (1996), 1477 – 1494