**Mycobacterium paratuberculosis Antibody Test Kit PARACHEK®**

**An in vitro diagnostic test kit for Detection of Antibodies to Mycobacterium paratuberculosis in Cattle, Sheep and Goats.**

184 Maximum Test Samples – Two (2) Microplate Test Kit
2760 Maximum Test Samples – Thirty (30) Microplate Test Kit

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Version 1.4_US

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**Introduction**

Johne’s disease is a chronic, debilitating enteritis of ruminants caused by infection with Mycobacterium paratuberculosis.

During the active stage of infection and prior to onset of clinical disease, animals generally develop antibodies to *M. paratuberculosis* antigens. Uninfected animals lack specific antibodies to *M. paratuberculosis*, but may have cross-reacting antibodies to other mycobacteria. These cross reacting antibodies can be removed by absorption of serum with *M. phlei* prior to commencement of the EIA.

This test is a solid phase, indirect enzyme immunoassay (EIA) to detect antibodies to *M. paratuberculosis* in serum and milk and may be used as a specific test for Johne’s disease in cattle. The test can also be used to detect *M. paratuberculosis* antibodies in serum from sheep and goats.

The test has been evaluated in animals from Johne’s disease-endemic and Johne’s disease-free regions of Australia and the United States. The test specificities were 96% or greater for cattle, sheep and goats. Calculations of specificity were affected by the history of the cattle herd under test. However, the EIA detected up to 80% of animals before onset of clinical disease and 60-65% of faecal shedders were EIA positive on or before first detection of *M. paratuberculosis* in their faeces. For sheep, the test sensitivity estimates were 38-44% and for goats, the sensitivity ranged from 65-88%.

The test should permit epidemiological studies and be a useful tool in the management and control of Johne’s disease in cattle, sheep and goats.

**References:**


(9) Whittington RJ, Eamons GJ, Cousins DV. Specificity of absorbed ELISA and agar gel immunodiffusion tests for paratuberculosis in goats with observations about use of these tests in infected goats. Aust Vet J 2003; 81: 71-6.

**Test Principle**

The test involves four separate stages:

**Stage 1**

Serum/milk samples are diluted and incubated in Green Diluent Buffer containing *M. phlei* to remove cross-reacting antibodies.

**Stage 2**

Diluted serum/milk samples are reacted with *M. paratuberculosis* antigens bound to a solid support. Unreacted proteins are removed by washing after a suitable incubation time.

**Stage 3**

Enzyme Substrate is added. The rate of conversion of substrate is proportional to the amount of bound immunoglobulin. Reaction is terminated after a suitable time and the amount of colour development estimated spectrophotometrically.

**Stage 4**

Enzyme Substrate is added. The rate of conversion of substrate is proportional to the amount of bound immunoglobulin. Reaction is terminated after a suitable time and the amount of colour development estimated spectrophotometrically.

**Kit Components**

**Product Numbers**

Two (2) Microplate Test Kit: 63307
Thirty (30) Microplate Test Kit: 63308

**Component 1**

Microtitre Plates coated with *M. paratuberculosis*. 63307: 2 x 96 well plates with lids 63308: 30 x 96 well plates with lids Ready for use.

**Component 2**

Positive Control. 63307: 1 x 0.75 ml 63308: 1 x 2.0 ml Contains 0.01% w/v thimerosal. Ready for use.

**Component 3**

Negative Control. 63307: 1 x 0.75 ml 63308: 1 x 2.0 ml Contains 0.01% w/v thimerosal. Ready for use.

**Component 4**

Green Diluent: Sample diluent buffer. Contains 0.01% w/v thimerosal. 63307: 1 x 100 ml 63308: 3 x 500 ml Ready for use.

**Component 5**

Wash Buffer – 20X Concentrate. 63307: 1 x 100 ml 63308: 2 x 500 ml Contains 0.01% w/v thimerosal. Dilute with deionised or distilled water.

**Component 6**

Conjugate – 100X Concentrate. (Horseradish peroxidase labelled anti-bovine Ig.) 63307: 1 x 0.5 ml 63308: 2 x 2 ml Contains 0.01% w/v thimerosal. Dilute with Blue Diluent.

**Component 7**

Blue Diluent: Conjugate diluent buffer. 63307: 1 x 30 ml 63308: 2 x 200 ml Contains 0.01% w/v thimerosal. Ready for use.

**Component 8**

Enzyme Substrate Buffer. 63307: 1 x 15 ml 63308: 2 x 200 ml Contains H2O2. Ready for use.

**Component 9**

Chromogen Solution – 100X Concentrate. 63307: 1 x 0.5 ml 63308: 2 x 2 ml Contains TMB in DMSO. Dilute in Enzyme Substrate Buffer.

**Component 10**

Enzyme Stopping Solution. 63307: 1 x 0 ml 63308: 1 x 200 ml (0.5M H2SO4) Ready for use.

**General Precautions**

1. **Laboratory Safety**
   - Correct laboratory procedures should be applied at all times.

2. **Chromogen Solution**
   - CAUTION: AVOID CONTACT WITH SKIN.
   - Handle chromogen solution with care since di-methyl sulphoxide (DMSO) is readily absorbed through the skin.

3. **Stopping Solution**
   - The enzyme stopping solution is a strong acid. Wipe up spills immediately. Flush the area of the spill with water. If the stopping solution contacts the skin or eyes, flush with copious quantities of water and seek medical attention.

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For in vitro veterinary diagnostic laboratory use only.

Store at 54±3°C

Product No.: 63307/63308

The protocol for the use of bovine milk samples has been optimized and validated by Antel BioSystems, Inc.
ENZYMES AND SUBSTRATES

Preparation of Reagents

1. Plates
   Allow plates to equilibrate to room temperature for at least 30 minutes before unsealing plastic pouch. Plates not required should be removed from frame and resealed in the plastic pouch along with desiccant. Frames and lids are reusable.

2. Positive and Negative Controls
   Bring to room temperature and mix each thoroughly. Use undiluted.

   NOTE: Up to 2 hours may be required to ensure a full bottle of Green Diluent has reached room temperature. If a shorter equilibration time is desired, an ambient temperature water bath must be used.

3. Blue Diluent
   Conjugate diluent buffer. Bring to room temperature and mix thoroughly. Use undiluted.

4. Conjugate 100X Concentrate
   Conjugate 100X Concentrate MUST be stored at 5°C at all times.

5. Preparation of Conjugate
   Bring Blue Diluent (Conjugate diluent buffer) to room temperature then mix thoroughly with Conjugate 100X Concentrate to make conjugate reagent ready for use. Suitable volumes are presented in the Conjugate and Substrate Preparation Table (Table 1).

   The working strength Conjugate Reagent should be used within 30 MINUTES OF PREPARATION and unused reagent immediately discarded. Return any unused Conjugate 100X Concentrate to 5±3°C immediately after use.

6. Wash Buffer
   Prepare working strength wash buffer by thoroughly mixing one part 20X concentrate with 19 parts distilled or distilled water. Working strength wash buffer may be stored at room temperature for up to 2 weeks.

7. Enzyme substrate solution
   Bring enzyme substrate reagents to room temperature for at least 30 minutes prior to mixing. Prepare enzyme substrate solution just prior to use by diluting the Chromogen Solution Concentrate in Enzyme Substrate Buffer as shown in the Conjugate and Substrate Preparation Table (Table 1). Enzyme substrate solution should be clear and colourless. Discard if blue coloration or any evidence of precipitation occurs. USE WITHIN 10 MINUTES OF PREPARATION.

   NOTE: If possible use plastic polypropylene disposable containers sterilised by irradiation to prepare the enzyme substrate solution.

   DO NOT USE POLYSTYRENE CONTAINERS OR PIPETTES.

   Procedural Notes

1. Equilibrate all reagents except the Conjugate 100X Concentrate to equilibrate to room temperature (22±3°C) for at least 30 minutes before use.

2. All kit components are to be stored at 5±3°C. Return to 5±3°C immediately after use. Working strength wash buffer may be stored at room temperature for up to 2 weeks.

3. The Conjugate 100X Concentrate must be left at 5±3°C at all times.

4. The assay has been started it should be completed without interruption.

5. Use a separate disposable tip for each sample to prevent cross contamination.

6. Test samples may be assayed in duplicate in adjacent wells.

7. The assay has been validated for serum and milk samples of cattle and for serum samples of sheep and goats. The protocol is optimized for fresh, frozen and preserved whole milk samples. Do not use colostrum or milk within 1 week of calving. A 1:1 dilution is applied only for the milk samples, the dilution of serum samples, Positive and Negative Controls remains 1/20.

8. Positive and Negative Controls must be assayed in duplicate. For convenience, controls may be located at the beginning of each plate (eg. Negative Controls in well A1 & B1, Positive Controls in wells C1 & D1).

   Test Procedure

1. Equilibrate all reagents except the Conjugate 100X Concentrate.

2. Add 25 µL of test serum and control samples to their appropriate tubes. If milk samples are tested, add 150 µL mixed whole milk to the appropriate tubes. Allow 1 tube per sample and 1 tube for each of the controls.

3. Add 475 µL (150 µL for milk samples) of Green Diluent to each of the tubes being very careful not to cross contaminate the tubes. If splashing or cross contamination does occur, discard the tubes and start again. Mix thoroughly either by pipetting up and down 3 to 5 times or by vortexing tubes several times.

4. Cover tubes and incubate at room temperature (22±3°C) for 30-60 minutes.

5. Add 100 µL of test and control samples to appropriate wells. Add control samples after the test samples have been added. Shake plate. Controls may be positioned anywhere on the plate (see “Procedural Notes”).

6. Cover each plate with a lid and incubate at room temperature (22±3°C) for 30 minutes. Conjugate 100X Concentrate should be diluted ready for use at the end of this incubation.

7. Shake out diluted serum/milk and control samples and wash trays 6 times at room temperature as follows. Fill wells with wash buffer taking care not to cross contaminate adjacent wells. Shake out wash fluid and repeat operation a further 5 times. After the sixth wash, tap trays face down several times on absorbent paper to remove as much remaining wash buffer as possible.

8. Add 100 µL of freshly prepared conjugate reagent to each well. Shake plate.

9. Cover each plate with a lid and incubate at room temperature for 30 minutes.

10. Wash plates as in Step 7.

   NOTE: The enzyme substrate solution is best prepared after this wash step.

11. Add 100 µL of freshly prepared enzyme substrate solution to each well.

12. Cover each plate with a lid, shake and incubate at room temperature. Optimal time of incubation is dependent on a number of variables. Time is best judged by reading the absorbance of the positive controls during colour development. A 620-650 nm filter must be used. Proceed to step 13 when the absorbance is between 0.35 and 0.40.

   NOTE: Gently mix the contents of plates by tapping to evenly distribute colour in Positive Control wells before estimating intensity as above. Protect from direct sunlight.

13. Add 50 µL of Enzyme Stopping Solution to each well, being careful not to transfer chromogen from well to well, then mix by gentle agitation.

   NOTE: The Enzyme Stopping Solution should be added to wells in the same order and at the same speed as the enzyme substrate solution.

14. Read the absorbance of each well using a 450 nm filter between 2 and 20 minutes after terminating the reaction. The absorbance values will then be used to calculate results.

   * If available, read plates using the monitored end point option (blank on air) of software, with the plate shaking option set to operate between each read.

   Validation of Test Performance

   The control results must be examined before the sample results can be interpreted.

   Determine the mean absorbance of Negative and Positive Controls.

   Acceptable range of means:

   - Negative control < 0.150

   NOTE: The duplicates must not vary by more than 0.040.

   - Positive control between 0.900 and 1.200

   NOTE: The positive control values must not deviate by more than 30% from its mean absorbance.

   The run is invalid if either of these criteria is not met and must be repeated.
Interpretation of Results

The cut-off value for a valid assay is the mean of the negative controls plus 0.100 for cattle, and 0.200 for sheep or goats, e.g. if the two negative controls were 0.055 and 0.085, the cut-off would be 0.070 + 0.100 = 0.170 for cattle, or 0.070 + 0.200 = 0.270 for sheep or goats. A positive result is a sample absorbance value greater than the cut-off value. A positive result indicates that the animal is likely to be infected with M. paratuberculosis and that there are potentially other infected animals within the herd.

A negative result on a single animal can only be interpreted when the paratuberculosis history and test results of the entire herd are known. Even though animals are generally infected as neonates, an immune response does not develop immediately. Current information suggests that seroconversion generally occurs before any clinical signs are apparent.

As with any biological test, this test may occasionally give a false positive or false negative result due to local conditions. A test should be interpreted in the context of all available clinical, historical and epidemiological information relevant to the animal(s) under test. Further confirmatory testing may be required in certain circumstances.

Responsibility for test interpretation and consequent animal husbandry decisions rests solely with the user, and any consulting veterinarian and appropriate animal health advisers or authorities.

Prionics AG accepts no responsibility for any loss or damage, however caused, arising from the interpretation of test results.

Technical Information

Follow the PARACHEK® (Johne’s Absorbed EIA) kit instructions as closely as possible, as any deviation may reduce the performance of the test and lead to erroneous results.

1. The kit instructions stipulate that 25 µL of serum is to be diluted with 475 µL of Green Diluent when assaying sera and 150 µL of milk is mixed with 150 µL of Green Diluent when testing milk samples. Any variation to this instruction may reduce the performance of the kit by introducing excess dilution errors.

2. Vortex mixing of conjugate in the Blue Diluent may reduce kit performance if it is done too vigorously. Vortex mixing should be done at a moderate speed, ‘stop-starting’ 3-4 times, in order to prevent or minimise frothing of the diluent. This approach should be used whenever vortex mixing any biological reagents.

However, not mixing enough may also lead to problems. Remember, it is impossible to over mix, as long as it is done gently. For accurate and reproducible results always ensure the conjugate is evenly mixed throughout the diluent.

3. Incubating plates (with or without a lid) directly on the bench is not recommended. The cold solid surface may act as a heat sink and lead to the phenomenon commonly known as ‘edge effects’. Plates should be incubated with a lid on, with the plate supported on an upturned, non-metal, test-tube rack so that air is free to circulate around the plate.

4. The kit instructions specify that when reagents are added to the plate, the plate should then be mixed by shaking. Deviation from this instruction may lower the reproducibility of the assay and lead to invalid results. After each reagent is added to the plate, the plate is to be placed on a plate shaker and shaken for one minute. The plate should also be similarly mixed before any optical reading is performed.

5. The use of a squeeze bottle to apply wash buffer to wash plates, instead of an EIA washer, may leave too much wash buffer on the plate wells after washing and may cause a significant number of bubbles. Excessive residual wash buffer may affect the performance of the substrate buffer. This may result in a significant reduction in the amount of colour developed by the EIA, manifested as an increased stop time. Because the amount of residual wash buffer varies well-to-well and plate-to-plate, the stop time will vary accordingly. The more wash buffer left in wells, the longer the stop time will be.

The following methodology is recommended:

Wash plates as detailed in the kit leaflet. After the last wash, tip out or aspirate the contents of the wells. Wipe plate dry with tissue paper, cover with lid and place upright on the bench. Excess wash buffer will drain to the bottom of the wells. Now, but not before, make up the appropriate volume of conjugate or substrate, which is ever applicable. Do not cause the conjugate to froth when mixing. It is critical that the conjugate is mixed thoroughly and done gently. If using a vortex mixer, use a relatively slow speed and ‘stop-start’ 3-4 times to ensure mixing is complete.

Now flick out the contents of the plate firmly, several times, until all the remaining wash buffer that has drained down into the bottom of the plate wells is removed. There should be virtually no wash buffer or bubbles visible in the wells. Gently wipe the top of the plate dry and proceed to add the conjugate or substrate. Place the plate on a shaker for about one minute to mix the well contents. Cover the plate with a lid and incubate, elevated off the bench surface, for the prescribed time. This is conveniently done by placing the plate on the grill bannister of an upturned plastic test-tube rack. This effectively maximises air circulation around the plate and minimises the so-called ‘edge effects’ phenomenon.

Further Reading


For a full list of references for the use of PARACHEK®, please contact: info@prionics.com

Parachek® is an approved diagnostic test for use in Australia’s National Johne’s disease Market Assurance Program.

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