**Mycobacterium paratuberculosis** Test Kit for Cattle

**PARACHEK® 2**

An *in vitro* diagnostic test kit for detection of antibodies to *Mycobacterium avium* ssp. *paratuberculosis* in serum of cattle, sheep and goat and milk of cattle

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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/plasma with *M. phlei* prior to commencement of the EIA.

The automated version of the PARACHEK® 2 is based on the original Johne’s absorbed EIA and shows the same high performance. It detects antibodies to *M. paratuberculosis* in serum, plasma and milk and may be used as a specific test for Johne’s disease in cattle. This original PARACHEK® 2 has been evaluated on animals from Johne’s disease- endemic and Johne’s disease-free regions of Australia and the United States. The test specificities were 99% or greater for cattle, sheep and goats. Calculations of sensitivity 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, goat and sheep.

**Test Principle**

The Method used is described in OIE Terrestrial Manual 2012, Vol. 1, chapter 2.1.11 (Paratuberculosis (Johne’s disease)).

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**

Antibodies in serum/milk samples are reacting with *M. paratuberculosis* in serum, plasma and milk and may be used as a specific test for Johne’s disease in cattle. This original PARACHEK® 2 has been evaluated on animals from Johne’s disease- endemic and Johne’s disease-free regions of Australia and the United States. The test specificities were 99% or greater for cattle, sheep and goats. Calculations of sensitivity 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, goat and sheep.

**Stage 3**

Conjugate (horseradish peroxidase labelled antibody IgG) reacts with immunoglobulins bound to the solid-phase antigen. Non binding conjugate is removed by washing after a suitable incubation time.

**Stage 4**

Enzyme Substrate is added. The rate of conversion of substrate is proportional to the amount of bound immunoglobulin. The reaction is stopped and the colour development is measured colorimetrically.

The 92 sample test in 1 plate can be finished in 90 minutes included sample pre-absorption.

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**Package Insert**

**Component 1**

**Microtitre Plates coated with *M. paratuberculosis***

- Component 3: 1 x 100 ml
- Component 2: 1 x 20 ml

**Ready for use**

**Component 2**

**Positive Control***

- Component 3: 1 x 0.75 ml
- Component 2: 1 x 1.0 ml

**Contains 0.01% w/v thimerosal. Ready for use.**

**Component 3**

**Negative Control***

- Component 3: 1 x 0.75 ml
- Component 2: 1 x 1.0 ml

**Contains 0.01% w/v thimerosal. Ready for use.**

**Component 4**

**Green Diluent: Sample diluent buffer***

- Component 3: 1 x 100 ml
- Component 2: 1 x 250 ml
- Component 1: 3 x 500 ml

**Contains 0.01% w/v thimerosal. Ready for use.**

**Component 5**

**Wash Buffer – 20x Concentrate***

- Component 1: 1 x 100 ml
- Component 2: 1 x 250 ml
- Component 3: 2 x 500 ml

**Contains 0.01% w/v thimerosal. Dilute with purified water.**

**Component 6**

**Conjugate – 100x Concentrate (Horseradish peroxidase labelled anti-bovine IgG)**

- Component 2: 1 x 0.5 ml
- Component 3: 1 x 0.75 ml
- Component 1: 2 x 2 ml

**Contains 0.01% w/v thimerosal. Dilute with Blue Diluent.**

**Component 7**

**Blue Diluent: Conjugate diluent buffer***

- Component 1: 1 x 30 ml
- Component 2: 1 x 75 ml
- Component 3: 2 x 200 ml

**Contains 0.01% w/v thimerosal. Ready for use.**

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**General Precautions**

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

2. **Enzyme 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.

**Equipment required but not provided**

- Accurate, replaceable-tip variable-volume pipettes (to deliver 475 µl, 25 µl and 10 to 12 µl)
- Graduated 1, 5 and 10 ml pipettes
- Measuring cylinders – 100 ml, 1 litre and 2 litres
- Suitable microtitre plate washer/dispenser
- Suitable microtitre plate shaker
- Multichannel pipette (to deliver 50 and 100 µl)
- Suitable microtitre plate reader. This reader MUST be fitted with filters to read at 450 nm and 620-650 nm.
- Dummy plate, used for sample dilution (e.g.clear colorless round bottom 96 well plates) or equivalent; non binding

**Preparation of Reagents**

1. **Microtitre Plates coated with *M. paratuberculosis***
   Allow plates to equilibrate to room temperature (22±3°C) for at least 30 minutes before unscrewing plastic pouch.

2. **Positive and Negative Controls**
   Bring to room temperature (22±3°C) and mix each thoroughly.

3. **Green Diluent**
   Sample diluent buffer. Bring to room temperature (22±3°C) and mix each thoroughly. (Ready-to-use).

4. **Blue Diluent**
   Conjugate diluent buffer. Bring to room temperature (22±3°C) and mix each thoroughly. (Ready-to-use).

5. **Conjugate 100x Concentrate**
   Conjugate 100x Concentrate MUST be stored at 5±3°C at all times.
6. Preparation of Conjugate
Bring Blue Diluent (Conjugate diluent buffer) to room temperature (22±3°C) then mix thoroughly with Conjugate 100x Concentrate to make conjugate reagent ready for use. Suitable volumes are presented in the Conjugate Preparation Table (Table 1).

Return Conjugate 100x Concentrate to 5±3°C immediately after use.

7. Wash Buffer – 20x Concentrate
Prepare working solution Wash Buffer by thoroughly mixing one part 20x Concentrate with 19 parts of purified water. Working solution Wash Buffer may be stored at room temperature (22±3°C) for up to 2 weeks.

8. Enzyme Substrate
Bring Enzyme Substrate to room temperature (22±3°C) for at least 30 minutes prior to use. Enzyme Substrate should be clear and colourless. Discard if blue coloration or any evidence of precipitation occurs. Tip the required volume into a vessel for use. Do not pipette in the supplied bottle, do not return unused solution to bottle.

DO NOT USE POLYSTYRENE CONTAINERS OR PIPETTES.

Any glassware used with the Enzyme Substrate should be rinsed thoroughly with 1N H2SO4 or HCl when using a liquid handler protect Enzyme Substrate from direct light and from evaporation.

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**CONJUGATE PREPARATION TABLE (TABLE 1)**

<table>
<thead>
<tr>
<th>Number of strips</th>
<th>Volume of concentrate</th>
<th>Volume of diluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10 µl</td>
<td>1 ml</td>
</tr>
<tr>
<td>2</td>
<td>20 µl</td>
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</tr>
<tr>
<td>3</td>
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<td>4</td>
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<td>7</td>
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<td>10</td>
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<tr>
<td>11</td>
<td>110 µl</td>
<td>11 ml</td>
</tr>
<tr>
<td>12</td>
<td>120 µl</td>
<td>12 ml</td>
</tr>
</tbody>
</table>

**Procedural Notes**

1. Allow all reagents except the Conjugate 100x Concentrate to equilibrate to room temperature (22±3°C) for at least 30 minutes before use. **NOTE:** Refer to special requirements for Green Diluent.

2. All kit components are to be stored at 5±3°C. Return to 5±3°C immediately after use. Wash Buffer working solution 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. Use a separate disposable tip for each sample to prevent cross contamination.

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

6. The assay has been validated for serum/plasma and milk sample. The protocol is optimized for fresh, frozen and preserved whole milk samples. Do not use colostrum or milk within 1 week of calving. A 1:2 dilution is applied only for the milk samples, the dilution of serum samples, Positive and Negative Controls remains at 1:20.

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

**Test Procedure**

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

2. Dilute serum samples 1:20 and milk samples 1:2 in Green Diluent in dummy plate, eg. 15 µl of test serum and control samples in 285 µl Green Diluent or duplicate test or 10 µl sample plus 190 µl Green Diluent for single test or 150 µl milk in 150 µl Green Diluent for duplicate test. Mix the sample and Green Diluent thoroughly either by pipetting up and down several times or by a microplate shaker for at least 1 minute with moderate speed. The reaction time of sample with Green Diluent may be from 1 minute up to 24 hours.

3. Transfer 100 µl of test and control samples from dummy plate to corresponding wells of the Microtitre Plate coated with M. paratuberculosis antigen.

4. Cover each plate with a lid and incubate at room temperature (22±3°C) for 30±30 minutes. Conjugate 100x Concentrate should be diluted ready for use at the end of the washing step. In the case of performance in automot, the conjugate may be used up to 8 hours after dilution at room temperature (22±3°C).

5. Wash plate 3x300 µl with Wash Buffer by using a plate washer with a suitable wash-program. If manual washing is applied, please take care not to cross contaminate adjacent wells. Shake out wash fluid as much as possible after each washing cycle. After the last washing cycle, tap trays face down several times on lint-free absorbent paper or similar materials to remove as much remaining wash buffer as possible.

6. Add 100 µl of freshly prepared conjugate solution to each well (see table 1).

7. Cover each plate with a lid and incubate at room temperature (22±3°C) for 30±30 minutes.

8. Wash plate as in Step 5.

9. Add 100 µl of Enzyme Substrate to each well.

10. Cover each plate with a lid, shake and incubate at room temperature (22±3°C) for 15-20 minutes.

11. Add 50 µl of Enzyme Stopping Solution to each 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.

12. Read the absorbance of each well using a 450 nm filter, use 620 nm as reference wavelength within 30 minutes after terminating the reaction. The absorbance values will then be used to calculate results.

**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.

Acceptance criteria

- The mean corrected value of Positive Control (PC) is greater than 0.500.

\[ OD_{PC} > 0.500 \]

- The mean corrected value of Positive Control (PC) is greater than 5 times the corrected value of Negative Control (NC)

\[ OD_{PC}/OD_{NC} > 5 \]

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

**Calculation of sample result**

\[ Sample\ %P (PP) = \frac{OD_{sample} - OD_{NC}}{OD_{PC} - OD_{NC}} \times 100 \]

\[ OD_{NC} = \text{mean value of Positive Control} \]

\[ OD_{PC} = \text{mean value of Negative Control} \]

The percent positivity (%P, PP) of PC is considered as 100%.

**Interpretation of Results**

- Results of bovine and ovine serum samples which are above or equal to the cut-off of 15 Percent Positivity (%P), or bovine milk samples which are above or equal to the cut-off of 10%P are considered as positive. 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.

- Results of bovine and ovine serum samples which are below the cut-off of 15 Percent Positivity (%P), or bovine milk samples which are below the cut-off of 10%P are considered as negative. A negative result on a single animal can only be interpreted when the paratuberculosis history and test results of the entire herd is 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® 2 (Johnie's Absorbed EIA) kit instructions as closely as possible. Any deviation may reduce the performance of the test and lead to erroneous results.

1. 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 that conjugate is homogenously mixed.

2. 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 at least one minute. The plate should also be similarly mixed before any optical reading is performed.

3. Blanking of EIA readers can be problematic. It appears that different readers blanked differently result in vastly different absorbance readings for the...
negative control. All readers should be blanked using a new unused plate or strip.

**General Remarks**

**Notice**
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**Safety Regulations / R&S Statements**

**Safety Regulations and R&S Statements**

**National Safety Regulations**

- **R&S Statements**

  **Component 1**
  **Microtitre Plates coated with M. paratuberculosis**
  Hazard Code: This product is not classified according to EU regulations.

  **Component 2**
  **Positive Control**
  Hazard Code: This product is not classified according to EU regulations.

  **Component 3**
  **Negative Control**
  Hazard Code: This product is not classified according to EU regulations.

  **Component 4**
  **Green Diluent: Sample diluent buffer**
  Hazard Code: This product is not classified according to EU regulations.

  **Component 5**
  **Wash Buffer – 20x Concentrate**
  Hazard Code: This product is not classified according to EU regulations.

  **Component 6**
  **Conjugate – 100x Concentrate**
  (Horseradish peroxidase labelled anti-bovine Ig)
  Hazard Code: This product is not classified according to EU regulations.

  **Component 7**
  **Blue Diluent: Conjugate diluent buffer**
  Hazard Code: This product is not classified according to EU regulations.

**Component 8**
**Enzyme Substrate**
Hazard Code: This product does not have to be labelled due to the calculation procedure of the “General Classification guideline for preparations of the EU” in the latest valid version.

**Component 9**
**Enzyme Stopping Solution**
Hazard Code: C Corrosive

**References**


5. 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: 71-5.

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