**Linnodee Leptospira *Hardjo* ELISA**

**Introduction**

Leptospirosis is an economically important zoonotic disease caused by a spirochaete bacteria of the genus *Leptospira*. Cattle-maintained leptospires of the serovar *hardjo* are the major cause of bovine leptospirosis\(^1\). This infection is responsible for considerable financial loss to the cattle industry as a consequence of agalactia, abortion, stillbirth, birth of weak calves and reduced fertility\(^2,3\). Moreover it also produces a renal-carrier state associated with long term urinary shedding\(^4,5\). Control schemes are in place in a number of countries, however, satisfactory methods for identifying carrier animals are not currently available\(^6\).

The Linnodee Bovine Leptospira ELISA kit is designed to detect the antibody response to a lipopolysaccharide (LPS) outer envelope epitope common to both *Leptospira borgpetersenii* serovar *Hardjo* (HB) and *Leptospira interrogans* serovar *Hardjo* (HP) in either sera or milk (bulk tank or individual animals). The monoclonal antibodies to the epitope used in the test have been shown to passively protect hamsters HB infection and to have high growth inhibition and leptospiricidal assay titres to HB\(^7\).

**Principle of the assay**

The assay employs a Double Antibody Sandwich Enzyme Linked Immunosorbent Assay (DAS-ELISA), in which Hardjo antigen is bound to wells pre-coated with Hardjo LPS specific monoclonal antibody. In the test system this antigen reacts with hardjo specific antibodies in the bovine milk/sera. Immobilised Hardjo antibodies are then detected by a monoclonal antibody linked to enzyme (HRP). This is quantified by measuring the amount of labelled detection antibody bound to the matrix using a chromogenic substrate (TMB-E) in a microplate spectrophotometer at an appropriate wavelength (450nm) with or without optical correction(620-700nm).

**Test Kit Contents**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-coated ELISA Plates</td>
<td>2</td>
<td>2-8°C</td>
</tr>
<tr>
<td>Wash Buffer (20 X concentrated)</td>
<td>1 x 100ml</td>
<td>2-8°C</td>
</tr>
<tr>
<td>Sample Diluent *</td>
<td>1 x 60ml</td>
<td>2-8°C</td>
</tr>
<tr>
<td>Positive Milk control*</td>
<td>2 x 2ml</td>
<td>2-8°C</td>
</tr>
<tr>
<td>Negative Milk control*</td>
<td>2 x 2ml</td>
<td>2-8°C</td>
</tr>
<tr>
<td>Positive Sera control*</td>
<td>1 x 200µl</td>
<td>2-8°C</td>
</tr>
<tr>
<td>Negative Sera control*</td>
<td>1 x 200µl</td>
<td>2-8°C</td>
</tr>
<tr>
<td>Substrate (TMB)*</td>
<td>1 x 40ml</td>
<td>2-8°C</td>
</tr>
<tr>
<td>Stop reagent (1M H(_2)SO(_4)*)</td>
<td>1 x 35ml</td>
<td>2-8°C</td>
</tr>
<tr>
<td>Peroxidase conjugated antibody (1000 x concentrate)</td>
<td>1 x 75µl</td>
<td>-20°C</td>
</tr>
</tbody>
</table>

* ready to use
Materials required but not provided
- Distilled water
- Adjustable single and multichannel micropipettes
- Single-use micropipette tips
- Incubator at 37°C, with or without shaker
- ELISA 96-well microplate reader equipped with 450nm filter (preferably with 630nm reference filter).
- Container for dilution of wash buffer solution
- Microtitre plate
- Plate sealers, universals, reservoirs, foil.

Precautions
- Care should be taken with test sera and milk, which could be microbiologically contaminated.
- Lab coat and gloves should be worn when handling reagents.
- Do not let the Sample Diluent / Wash Buffer 20 x / Substrate / Stop Solution come in contact with the skin, eyes or mouth.
- Good Laboratory Practice should be employed to avoid cross contamination of samples and reagents.
- Do not use kit components past their expiry dates.
- Do not mix kit components or instruction manuals from different batches or source.
- For in vitro veterinary use only.

Storage and Stability
The peroxidase conjugated antibody should be stored at -20°C and can be used directly from storage. The conjugate contains a cryoprotectant, which prevents its deterioration with repeated thawing and refreezing. All other kit reagents should be stored at 2-8°C and allowed to come to room temperature (20°C- 25°C) before use. The concentrated conjugate and control milk and sera should be returned to storage immediately after use.
Kit components will remain stable until the date of expiry (as indicated on the box) when stored under the recommended conditions.

Preparation
- Determine the total number of samples and controls to be tested. At least 3 replicates of each control (milk and/or sera) are recommended for each test run. For the strip kit, remove any strips not required from the frame and store in the resealable bag provided at 4°C.
- Crystals in the concentrated wash buffer can be dissolved by heating to 37°C. Dilute the wash buffer 1:20 in distilled water.
- Dilute the concentrated conjugate 1:1000 in diluted wash buffer just before use. The diluted conjugate is light sensitive and should be protected from exposure to light.

Incubation
The incubation times outlined below are suitable only when using a plate shaker at 37°C. For incubation without shaking, the sample incubation period will be one hour and the conjugate incubation period 40 minutes.

Directions for Washing
For each washing step*, wash the test wells with at least 200μl/well of diluted wash buffer. A wash bottle may be used, however care should be taken to avoid cross contamination. If using an automatic plate washer, it is
advisable to refer to the operating instructions. Following the final wash, remove residual wash buffer by inverting the plate and blotting firmly on absorbent paper.

**Test procedure**

The use of a dummy plate for more than ten samples (milk/sera) is strongly advised. Reference milk can be added directly to the test plate.

1. **Sample incubation**

   1.1 **Milk**
   - Add 150μl of undiluted milk sample to each well of dummy plate.
   - Using a multichannel pipette transfer 100μl of samples to the test wells.

   1.2 **Sera**
   - Dilute sample and control sera samples 1:50 in sample diluent
     e.g. for 200μl = 4μl serum to 196μl diluent on dummy plate
   - Using a multichannel pipette mix the contents of the wells and transfer 100μl to the test wells

   1.3 **Milk and Sera Incubation**
   - Cover the wells with a plate sealer and incubate at 37°C for 40 minutes with shaking.
   - Wash the wells with wash buffer four times as described above *.

2. **Conjugate incubation**

   - Add 100μl of the diluted conjugate to each well.
   - Cover the wells with a plate sealer and incubate in the dark at 37°C for 30 minutes with shaking.
   - Wash the wells with wash buffer four times *.

3. **Substrate incubation**

   - Add 100μl of the substrate to each well.
   - Incubate the strips/plate in the dark at room temperature for 10 minutes.
   - Add 50μl of stop solution to each well at the same order as the substrate was added.
   - Read at a wavelength of 450nm or with a corrected OD using a reference filter (e.g. 630nm)

**Assay Protocol Quick Reference**

- Sample / control 100μl/well
- Conjugate 100μl/well
- Substrate 100μl/well
- Substrate + Stop 50μl/well
- Plate Reader
Calculations for Milk & Sera

The S/P ratio (Sample value related to Positive Control value) can be calculated using the following formula:

\[
\text{S/P ratio} = \frac{\text{Mean Sample OD} - \text{Mean Negative control OD}}{\text{Mean Positive control OD} - \text{Mean Negative control OD}}
\]

The S/P ratio must be calculated using the respective controls for the sample type eg. use milk controls for milk samples.

**Interpretation of results**

**Interpretation for Bulk Milk**

<table>
<thead>
<tr>
<th>Result</th>
<th>If the ratio is:</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEGATIVE</td>
<td>≤ 0.03</td>
<td>Naïve and/or unvaccinated</td>
</tr>
<tr>
<td>LOW POSITIVE</td>
<td>≥ 0.04 ≤ 0.10</td>
<td>A few seropositive cows</td>
</tr>
<tr>
<td>MEDIUM POSITIVE</td>
<td>&gt; 0.10 ≤ 0.50</td>
<td>Mix of infected and susceptible animals</td>
</tr>
<tr>
<td>HIGH POSITIVE</td>
<td>&gt; 0.50</td>
<td>Heavily infected/vaccinated</td>
</tr>
</tbody>
</table>

**Interpretation for individual animal milk/sera**

<table>
<thead>
<tr>
<th>Result</th>
<th>If the ratio is:</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEGATIVE</td>
<td>Sera ≤ 0.05</td>
<td>Naïve and/or unvaccinated</td>
</tr>
<tr>
<td></td>
<td>Milk ≤ 0.03</td>
<td></td>
</tr>
<tr>
<td>INCONCLUSIVE</td>
<td>Sera ≥ 0.06 ≤ 0.12</td>
<td>Retest is recommended after a few weeks.</td>
</tr>
<tr>
<td></td>
<td>Milk ≥ 0.04 ≤ 0.10</td>
<td>If the sample is inconclusive after retest, the animal can be considered negative depending on the herd history.</td>
</tr>
<tr>
<td>POSITIVE</td>
<td>Sera &gt; 0.12</td>
<td>Exposed to infection/vaccinated</td>
</tr>
<tr>
<td></td>
<td>Milk &gt; 0.10</td>
<td></td>
</tr>
</tbody>
</table>

**For example,**

<table>
<thead>
<tr>
<th>Positive Control OD</th>
<th>Negative Control OD</th>
<th>Sample Type</th>
<th>Mean OD</th>
<th>Corrected OD</th>
<th>Ratio</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk Pos control 1</td>
<td>1.258</td>
<td>Milk Neg control 1</td>
<td>0.009</td>
<td>0.019</td>
<td>0.00</td>
<td>Negative</td>
</tr>
<tr>
<td>Milk Pos control 2</td>
<td>1.278</td>
<td>Milk Neg control 2</td>
<td>0.011</td>
<td>0.106</td>
<td>0.07</td>
<td>Inconclusive</td>
</tr>
<tr>
<td>Milk Pos control 3</td>
<td>1.364</td>
<td>Milk Neg control 3</td>
<td>0.021</td>
<td>0.180</td>
<td>0.13</td>
<td>Positive</td>
</tr>
<tr>
<td>Mean Milk Pos control</td>
<td>1.300</td>
<td>Mean Milk Neg control</td>
<td>0.014</td>
<td>0.049</td>
<td>0.03</td>
<td>Negative</td>
</tr>
<tr>
<td>Corrected Milk Pos control OD</td>
<td>1.286</td>
<td>Bulk Milk Sample 4</td>
<td>0.148</td>
<td>0.134</td>
<td>0.10</td>
<td>Low Positive</td>
</tr>
<tr>
<td>Sera Pos control 1</td>
<td>1.524</td>
<td>Sera Neg control 1</td>
<td>0.032</td>
<td>0.245</td>
<td>0.18</td>
<td>Medium Positive</td>
</tr>
<tr>
<td>Sera Pos control 2</td>
<td>1.568</td>
<td>Sera Neg control 2</td>
<td>0.028</td>
<td>1.124</td>
<td>0.86</td>
<td>High Positive</td>
</tr>
<tr>
<td>Sera Pos control 3</td>
<td>1.628</td>
<td>Sera Neg control 3</td>
<td>0.042</td>
<td>0.333</td>
<td>0.00</td>
<td>Negative</td>
</tr>
<tr>
<td>Mean Sera Pos control</td>
<td>1.573</td>
<td>Mean Sera Neg control</td>
<td>0.034</td>
<td>0.206</td>
<td>0.11</td>
<td>Inconclusive</td>
</tr>
<tr>
<td>Corrected Sera Pos control OD</td>
<td>1.539</td>
<td>Individual Sera Sample 10</td>
<td>0.242</td>
<td>0.208</td>
<td>0.14</td>
<td>Positive</td>
</tr>
</tbody>
</table>

Milk sample ratio = (mean milk sample OD - mean milk neg control OD) / (mean milk pos control OD - mean milk neg control OD)

Sera sample ratio = (mean sera sample OD - mean sera neg control OD) / (mean sera pos control OD - mean sera neg control OD)
Quality Control

- If any of the three positive or negative control ODs differ by more than 20% from the mean reference OD, discard the individual errant OD result.

- The test is invalid if the ratio is
  - greater than 0.10 for the negative milk control and 0.20 for the negative serum control respectively whenever a dual wavelength is used.
  - greater than 0.15 for the negative milk control and 0.25 for the negative serum control respectively whenever single wavelength is used.

Technical Hints

- Substrate solution should remain colourless until added to the plate
- Add stop solution to the plate in the same order as substrate solution
- Substrate solution in the wells should change from colourless to gradations of yellow/orange
- Avoid foaming of solutions during mixing
- Use separate reservoirs for each reagent
- Take only required volume of reagent for test in the reservoirs, especially substrate. Do not return unused substrate to the source bottle.
- Approximately 1 ml of the ready to use/diluted reagent will be required for one strip, while 10 ml will be required for 1 plate.
- Pre rinse the pipette tips with reagent to compensate for surface tension
- Milk samples need to be collected well below the creamy layer on top. Alternatively, the milk can be centrifuged at 3000rpm for 15 minutes to avoid the lipid interference.

Troubleshooting

1. Low absorbance
   - Incorrect dilutions or pipetting error
   - Improper incubation time
   - Substrate not in room temperature
   - Incorrect reagents used
   - Kit components or reagents contaminated/expired

2. High absorbance
   - Cross contamination from other samples or positive controls
   - Incorrect dilutions or pipetting error
   - Improper washing
   - Wrong filter on plate reader
   - Contaminated buffers/enzyme substrate
   - Improper Incubation time
   - Reuse of plate sealers, reservoirs or pipette tips

3. Poor Duplicates
   - Poor mixing of samples
   - Incorrect dilutions/pipetting error
   - Inefficient washing
   - Cross contamination from other samples or positive controls
   - Reuse of plate sealers, reservoirs or pipette tips
4. **All wells are Positive**
   - Contaminated buffers/substrate
   - Inefficient washing
   - Technical error

5. **All wells are Negative/Blank**
   - Procedure not followed correctly
   - Contaminated buffers/substrate
   - Contamination / Improper storage of conjugate
   - Kit reagents/ components expired

**Additional Information**

The cut off values may require refinement for the local population to obtain an acceptable range of false positive and negative results. In-house calibrators can be maintained to check the assay performance.

**References**


