Linnodee Porcine Leptospirosis Kit

Introduction
Leptospira interrogans  serovar Bratislava has emerged as a major pig maintained infection worldwide\(^1\). Infections can result in abortions, full-term birth of dead or weak piglets of reduced viability or infertility. Leptospires persist in kidneys and genital tracts of carrier swine and are excreted in urine and genital fluids. Diagnosis of leptospirosis in pigs is extremely difficult, and is usually based on serology using the microscopic agglutination test (MAT), which has a low sensitivity for detecting past or current infection\(^2,3\).

Principle of the assay
A competitive ELISA (cELISA) has been developed which incorporates a monoclonal antibody to Bratislava specific lipopolysaccharide (LPS). The monoclonal did not cross react with antigen preparations from 11 other serovars, therefore increasing the specificity of the cELISA. The monoclonal antibodies compete with anti-LPS antibodies in serum. The specific porcine antibodies, if present, bind to the Bratislava antigen coated plate, inhibiting binding of the monoclonal antibody. The % inhibition of the porcine sera is measured by the detection of the monoclonal using another labelled antibody. This is then allowed to act on a substrate and the colour developed can be measured using a microplate reader.

Test Kit Contents

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>96 well ELISA plates</td>
<td>5</td>
<td>2-8°C</td>
</tr>
<tr>
<td>Positive Sera control*</td>
<td>400µl</td>
<td>2-8°C</td>
</tr>
<tr>
<td>Negative Sera control*</td>
<td>400µl</td>
<td>2-8°C</td>
</tr>
<tr>
<td>Coating Buffer</td>
<td>60ml</td>
<td>2-8°C</td>
</tr>
<tr>
<td>Sample Diluent</td>
<td>120ml</td>
<td>2-8°C</td>
</tr>
<tr>
<td>Concentrated Wash Buffer (15x)</td>
<td>130ml</td>
<td>2-8°C</td>
</tr>
<tr>
<td>Concentrated Blocking Buffer (2x)</td>
<td>90ml</td>
<td>2-8°C</td>
</tr>
<tr>
<td>L. Bratislava antigen</td>
<td>650 µl</td>
<td>2-8°C</td>
</tr>
<tr>
<td>Anti-L. Bratislava monoclonal antibody</td>
<td>120 µl</td>
<td>2-8°C</td>
</tr>
<tr>
<td>Anti-mouse peroxidase conjugated antibody concentrate</td>
<td>30 µl</td>
<td>2-8°C</td>
</tr>
<tr>
<td>Substrate (1M H(_2)SO(_4))</td>
<td>60ml</td>
<td>2-8°C</td>
</tr>
<tr>
<td>Stop Reagent (1M H(_2)SO(_4))</td>
<td>35ml</td>
<td>2-8°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 and blocking buffer solution
  - Microtitre dummy 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 15 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 ELISA plates can be stored at room temperature; all other kit reagents should be stored at 2-8°C. The concentrated conjugate and control milk and sera should be returned to storage immediately after use. When stored under the correct conditions, the kit components will remain stable until the date of expiry (as indicated on the box).

Preparation
- Remove the individual kit components, including the diluted wash and blocking buffers, allow them to warm to room temperature (20°C – 25°C) for at least 1 hr.
- 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.
- Crystals in the concentrated wash buffer can be dissolved by heating to 37°C with stirring. Dilute the wash buffer 1:15 in distilled water.
- Dilute the anti-L.Bratislava monoclonal antibody immediately before use in blocking buffer (x1) by 1/600.
- Dilute the L.Bratislava antigen immediately before use in coating buffer by 1/100.
- Dilute the blocking buffer 1:2 in distilled water (approximately 36mls of blocking buffer is needed per plate, here add 18mls 2 x conc. blocking buffer to 18mls distilled water).
- Dilute the concentrated conjugate 1:4000 immediately before use in diluted blocking buffer. The diluted conjugate is light sensitive and should be protected from exposure to light.
- Coating buffer, sample diluent, substrate and stop reagent are ready to use.
Incubation
The incubation times outlined below are suitable only when using a plate shaker at 37°C.

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 (sera) is strongly advised.

1. Coating of Antigen
   - Coat the plate with 1/100 dilution of L. Bratislava antigen in coating buffer, 100µl per well.
   - Cover the wells with a plate sealer and incubate at 37°C with agitation for 30 minutes.
   - Wash the plate 3 times with 200µl/well Wash Buffer.

2. Blocking of the plate
   - Coat the plate with blocking buffer, 100µl per well.
   - Cover the wells with a plate sealer and incubate at 37°C with agitation for 30 minutes
   - Wash the plate 3 times with 200µl/well Wash Buffer.

3. Sample incubation
   - 100µl volumes per well of 1/10 dilutions of sera are used.
   - Positive and negative controls must be run in duplicate each time the assay is run, they can be added directly to the plate (i.e. 90µl sample diluent on plate + 10µL control sera).
   - 100µl sample diluent only is added to 4 wells, these wells are used as the control OD, (= 0% Inhibition)
   - Dilute sera 1 in 10 in sample diluent, e.g. add 180µl sample diluent to each well of a dummy plate + 20µl sample. Using a multichannel pipette mix well and then transfer 100µl of the samples to the test plate.
   - Cover the wells with a plate sealer and incubate at 37°C with agitation for 45 minutes.
   - Wash the plate 4 times with 200µl/well wash buffer.

4. Incubation with Monoclonal Antibody
   - Add 100µl of 1/600 dilution of the anti-L. Bratislava monoclonal antibody in blocking buffer to all wells.
   - Cover the wells with a plate sealer and incubate at 37°C with agitation for 30 minutes.
   - Wash the plate 3 times with 200µl/well wash buffer.
5. **Conjugate incubation**

- Add 100µl of 1/4000 dilution of peroxidase conjugate in blocking buffer to all wells.
- Cover the wells with a plate sealer and incubate at 37°C with agitation for 30 minutes.
- Wash the plate 3 times with 200µl/well wash buffer.

6. **Substrate incubation**

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

**Calculation and Interpretation of Results**

*The results of the cELISA are expressed as % inhibition of the porcine sera:*

\[
\text{% Inhibition} = \left(\frac{\text{Control OD} - \text{Test Serum OD}}{\text{Control OD}}\right) \times 100
\]

The result is considered positive if % Inhibition > 40%
The result is considered negative if % Inhibition ≤ 40%

**Quality Control**

- If any of the 4 control ODs differ by more than 10% from the mean Control OD discard the value.
- If % inhibition of mean negative control > 40% the test should be considered invalid.
- If % inhibition of mean positive control < 70% the test should be considered invalid

**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.
- 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.

**Troubleshooting**

1. **Low absorbance**

- Incorrect dilutions or pipetting error
- Improper incubation time
- Substrate not in room temperature
- Incorrect reagents used
2. **High absorbance**
   - Kit components or reagents contaminated/expired
   - Cross contamination from other samples or negative 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 Negative/Blank**
   - Contaminated buffers/substrate
   - Inefficient washing
   - Technical error

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

**References**


