**BOVINE LACTOFERRIN ELISA**

*Life Diagnostics, Inc.*, Catalog Number: LF-11

**INTRODUCTION**
Lactoferrin is a non-heme iron binding glycoprotein found in milk, other secretory fluids and blood. As a component of host defense, it has antimicrobial and anti-inflammatory activity. It is an excellent biomarker of mastitis in cattle (refs 1&2). Lactoferrin levels may range from less than 0.05 mg/ml in normal milk to more than 8 mg/ml in milk from animals with mastitis (refs 1&2).

**PRINCIPLE OF THE ASSAY**
The bovine lactoferrin test kit is based on a solid phase enzyme-linked immunosorbent assay (ELISA). The assay uses affinity purified anti-bovine lactoferrin antibodies for solid phase (microtiter wells) immobilization and horseradish peroxidase (HRP) conjugated anti-bovine lactoferrin antibodies for detection. The test sample is diluted and incubated in the microtiter wells for 45 minutes. The microtiter wells are subsequently washed, and HRP conjugate is added and incubated for 45 minutes. This results in lactoferrin molecules being sandwiched between the immobilization and detection antibodies. The wells are then washed to remove unbound HRP-labeled antibodies, and TMB Reagent is added and incubated for 20 minutes at room temperature. This results in the development of a blue color. Color development is stopped by the addition of Stop Solution, changing the color to yellow, and optical density is measured spectrophotometrically at 450 nm. The concentration of lactoferrin is proportional to the optical density of the test sample.

**MATERIALS AND COMPONENTS**

*Materials provided with the kit:*
- Anti-Bovine Lactoferrin Coated Microtiter Plate (provided as 12 detachable strips of 8 wells)
- Enzyme Conjugate Reagent, 11 ml
- Reference standard (lyophilized)
- 20x Wash Buffer, 50 ml
- 10x Diluent, 25 ml
- TMB Reagent (One-Step), 11 ml
- Stop Solution (1N HCl), 11 ml

*Materials required but not provided:*
- Precision pipettes and tips
- Distilled or deionized water
- Polypropylene or glass tubes
- Vortex mixer
- Absorbent paper or paper towels
- Micro-plate incubator/shaker with mixing speed of ~150 rpm
- Plate washer
- Plate reader with an optical density range of 0-4 at 450nm
- Graph paper (PC graphing software is recommended)

**STORAGE**
The kit should be stored at 2-8°C, and the microtiter plate should be kept in a sealed bag with desiccant to minimize exposure to damp air. Test kits will remain stable for six months from the date of purchase provided that the components are stored as described above.

**WASH SOLUTION PREPARATION**
The wash solution is provided as a 20x stock. Prior to use, dilute the contents of the bottle (50 ml) with 950 ml of distilled or deionized water.

**DILUENT PREPARATION**
The diluent is provided as a 10x stock. Prior to use, estimate the final volume of diluent required for your assay and dilute one (1) volume of the 10x stock with nine (9) volumes of distilled or deionized water.

**STANDARD PREPARATION**
1. The bovine lactoferrin standard is provided as a lyophilized stock. Add the volume of distilled or deionized water indicated on the vial label and mix gently until dissolved *(the reconstituted standard should be aliquoted and frozen at -20°C if additional use is intended)*.
2. Label 7 polypropylene or glass tubes as 100, 50, 25, 12.5, 6.25, 3.13, and 1.56 ng/ml
3. In the tube labeled 100 ng/ml, prepare the 100 ng/ml standard by diluting the reconstituted stock with 1x diluent as detailed on the standard label.
4. Dispense 250 μl of 1x diluent into the tubes labeled 50, 25, 12.5, 6.25, 3.13, and 1.56 ng/ml.
5. Prepare a 50 ng/ml standard by diluting and mixing 250 μl of the 100 ng/ml standard with the 250 μl of diluent in the tube labeled 50 ng/ml.
6. Similarly prepare the 25, 12.5, 6.25, 3.13, and 1.56 ng/ml standards by serial dilution.

**SAMPLE PREPARATION**
*Milk:* For optimum results, milk should be processed to skim milk or whey. Skim milk can be obtained by centrifugation of milk at >3000g for ~15 min at 4°C. Whey is prepared by adjustment of skim milk to pH 4.5 with glacial acetic acid followed by centrifugation and readjustment of the supernatant pH to 6.5 – 7.5 using 5 M NaOH. In order to obtain values within the range of the standard curve, we suggest that samples be diluted 10,000 fold using the following procedure for each sample to be tested:
- Dispense 495 μl of 1x diluent into two tubes.
- Pipette and mix 5.0 μl of the sample into the first tube and mix. This provides a 100 fold diluted sample.
- Mix 5.0 μl of the 100 fold diluted sample with the 495 μl of diluent in the second tube. This provides a 10,000 fold dilution of the sample.

*Serum:* Lactoferrin levels in normal serum are ~100 ng/ml. Samples must be diluted 20-fold or more prior to assay in order to avoid undesirable matrix effects.

**ASSAY PROCEDURE**
1. Secure the desired number of coated wells in the holder.
2. Dispense 100 μl of standards and diluted samples into the wells *(we recommend that samples be tested in duplicate)*.
3. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 45 minutes.
4. Wash and empty the microtiter wells 5 times with 1x wash solution. This should preferably be performed using a plate
washer (400 μl/well). If a plate washer is not available, use a squirt bottle. The entire wash procedure should be performed as quickly as possible.
5. Strike the wells sharply onto absorbent paper or paper towels to remove all residual droplets.
6. Add 100 μl of enzyme conjugate reagent into each well.
7. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 45 minutes.
8. Wash as detailed in steps 4 to 5 above.
9. Dispense 100 μl of TMB Reagent into each well.
10. Gently mix on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 20 minutes.
11. Stop the reaction by adding 100 μl of Stop Solution to each well.
12. Gently mix. It is important to make sure that all the blue color changes to yellow.
13. Read the optical density at 450 nm with a microtiter plate reader within 5 minutes. In the event that the standards or samples exceed the absorbance range of your plate reader, optical density may be determined at 405 nm.

**CALCULATION OF RESULTS**
1. Calculate the average absorbance values ($A_{450}$) for each set of reference standards and samples.
2. Construct a standard curve by plotting the mean absorbance obtained from each reference standard against its concentration in ng/ml on linear graph paper, with absorbance values on the vertical or Y-axis and concentrations on the horizontal or X-axis. Please note that the 1.56 – 50 ng/ml data points are optimum for curve fitting. On the discretion of the user, the 100 ng/ml standard can be omitted.
3. Using the mean absorbance value for each sample, determine the corresponding concentration of lactoferrin in ng/ml from the standard curve.
4. Multiply the derived concentration by the dilution factor to determine the actual concentration of lactoferrin in the serum/plasma sample.
5. PC graphing software should preferably be used for the above steps. We find that a second order polynomial model gives a good fit of the standard data.
6. If the $A_{450}$ values of samples fall outside the standard curve when tested at a 10,000 fold dilution, samples should be diluted appropriately and re-tested.

**TYPICAL STANDARD CURVE**
A typical standard curve with optical density readings at 450nm on the Y-axis against lactoferrin concentrations on the X-axis is shown below. This curve is for the purpose of illustration only and should not be used to calculate unknowns. Each user should obtain his or her data and standard curve in each experiment.

<table>
<thead>
<tr>
<th>Lactoferrin (ng/ml)</th>
<th>$A_{450}$</th>
</tr>
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<tbody>
<tr>
<td>100</td>
<td>3.641</td>
</tr>
<tr>
<td>50</td>
<td>2.553</td>
</tr>
<tr>
<td>25</td>
<td>1.638</td>
</tr>
<tr>
<td>12.5</td>
<td>0.967</td>
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<tr>
<td>6.25</td>
<td>0.606</td>
</tr>
<tr>
<td>3.13</td>
<td>0.408</td>
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<tr>
<td>1.56</td>
<td>0.270</td>
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</tbody>
</table>

**LIMITATIONS OF THE PROCEDURE**
1. Reliable and reproducible results will be obtained when the assay procedure is carried out with a complete understanding of and in accordance with the instructions detailed above.
2. All reagents should be allowed to reach room temperature (18-25°C) before use.
3. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.

**REFERENCES**

Rev 041515NC
For technical assistance please email us at techsupport@lifediagnostics.com