Bovine lactoferrin

ELISA kit for the quantitative determination of Bovine lactoferrin
Competitive test for blood sera, milk and colostrum
Diagnostic test for cattle
Monowell

I - INTRODUCTION

Lactoferrin is a glycoprotein found in most external secretions: milk, saliva, tears, etc., and in polymorphonuclear leucocytes. Its molecular mass is 77 kDa and it contains 2 binding sites for one ferric ion (Fe+++). This capacity to fix iron partly explains lactoferrin's antibacterial activity. At the present time, other activities of this protein, such as its effect on cell growth, its role in iron transport and its immunostimulating action have been demonstrated.
During the inflammatory reaction, lactoferrin is liberated in the extracellular medium from secondary granules of the neutrophils. It is possible to determine the neutrophils' activation index from the extracellular lactoferrin concentration. The Bio-X Bovine Lactoferrin Elisa Kit allows the determination of the lactoferrin concentration in various biological liquids (milk, colostrum, blood serum, etc.) and in industrial preparations (milk powders, toothpastes, artificial saliva, etc.)
The ELISA technique is rapid and reliable and is particularly suited to the analysis of large numbers of samples.

II – PRINCIPLE OF THE TEST

The test uses microplates that have been sensitized with an anti-bovine lactoferrin specific polyclonal antibody. Cross-reactions with lactoferrin from other species (notably human lactoferrin) are possible. The pre-diluted samples are deposited at the same time as the conjugate in the plate's microwells. The conjugate is composed of purified bovine lactoferrin that is chemically bound to peroxidase. To get quantitative readings, it is necessary to plot a dilution curve from a known concentration of bovine lactoferrin. This calibration reference is supplied in the kit. After one hour's incubation at 21°C +/- 3°C the plate is washed and the chromogen (tetramethylbenzidine) is added. This chromogen has the advantage of being more sensitive than the other peroxidase chromogens and not being carcinogenic.
Comparing the signals recorded for the unknown samples with the calibrator's dilution curve provides an easy way to determine the lactoferrin concentration in the initial sample.

III - COMPOSITION OF THE KIT

- **Microplates**: Two 96-well microtitration plates. The whole plate is sensitized by anti-bovine lactoferrin-specific antibodies.
- **Washing solution**: One 100-ml bottle of 20x concentrated washing solution. The solution crystallizes spontaneously when cold. If only part of the solution is to be used, bring the bottle to 21°C +/- 3°C until disappearance of all crystals. Mix the solution well and remove the necessary volume. Dilute the buffer 1:20 with distilled or demineralized water. Store the diluted solution between +2°C and +8°C.
- **Dilution buffer**: One 30 ml bottle colored buffer for diluting conjugate. This reagent is ready to use.
- **Conjugate**: One vial of 50x concentrated lactoferrin-peroxidase conjugate (horseradish peroxidase-labelled bovine lactoferrin).
- **Lactoferrin standard**: 2 bottles containing the bovine lactoferrin standard. Each bottle contains approximately 80 mg of bovine lactoferrin.
- **Single component TMB:** One 25 ml bottle. The reagent is ready to use.
- **Stop solution**: One 15-ml bottle of the 1 *M* phosphoric acid stop solution.

	BIO K 158/2
Microplates	2
Washing solution	1 X 100 ml (20x)
Colored Dilution buffer	1 X 30 ml (1X)
Conjugate	1 X 0,5 ml (50x)
Lactoferrin Standard	2 freeze-dried vials
Single component TMB	1 X 25 ml (1x)
Stop solution	1 X 15 ml (1x)

IV - ADDITIONAL MATERIALS AND EQUIPMENT REQUIRED

Distilled water, graduated cylinders, beakers, volumetric flask 1l, plastic tubes, tube rack, dispenser tips, reagent reservoir for multichannel pipettes, lid, adhesive for microplates, graduated automatic (mono- and multichannel) pipettes, microplate reader, and microplate washer and shaker (optional)

V - PRECAUTIONS FOR USE

- This test may be used for “in vitro” diagnosis only. It is strictly for veterinary use.
- The reagents must be kept between +2°C and +8°C. The reagents cannot be guaranteed if the shelf-life dates have expired or if they have not been kept under the conditions described in this insert.
- The concentrated wash solution may be stored at room temperature. Once diluted, this solution remain stable for six weeks if kept between +2°C and +8°C.
- Unused strips must be stored immediately in the aluminium envelope, taking care to keep the desiccant dry and the envelope’s seal airtight. If these precautions are taken, the strips’ activity can be conserved up to the kit’s shelf-life date.
- Do not use reagents from other kits.
- The quality of the water used to prepare the various solutions is of the utmost importance. Do not use water that may contain oxidants (e.g., sodium hypochlorite) or heavy metal salts, as these substances can react with the chromogen.
- Discard all solutions contaminated with bacteria or fungi.
- The stop solution contains 1 M phosphoric acid. Handle it carefully.
- All materials and disposable equipment that come in contact with the samples must be considered potentially infectious and be disposed of in compliance with the legislation in force in the country.
- To guarantee the reliability of the results, one must follow the protocol to the letter. Special care must be taken in observing the incubation times and temperatures, as well as measuring the volumes and dilutions accurately.

VI – PROCEDURE

1- Bring all the reagents to 21°C +/- 3°C before use.
2- Dilute the concentrated washing solution 20 fold in distilled water. Be sure that all crystals have disappeared before dilution.
Keep this solution between +2°C and + 8°C when not used.
3- Prepare the standard solution of lactoferrin as follows: weigh precisely 50 mgr of powder in a small beaker and dissolve it in 50 ml of PBS using a magnetic stirrer. When the powder is entirely dissolved, transfer the solution to a 1-liter volumetric flask. Adjust the volume to 1 liter with PBS. The lower part of the meniscus must be tangent to the line on the volumetric flask. Close the volumetric flask and mix carefully. This solution is now at a concentration of 50µg/ml. The precision of the ELISA test partly depends on the care with which the standard solution of lactoferrin is prepared.
Dilute the standard solution in PBS as indicated in the table below. In order to do the standard curve, it is advisable to use precision equipment such as Hamilton syringes.

	Dilution	Concentration
Stock solution	1/1	50 µg/ml
1st dilution	1/5	10000 ng/ml
2nd dilution	1/7.5	6667ng/ml
3rd dilution	1/11.25	4444 ng/ml
4th dilution	1/16.88	2963 ng/ml
5th dilution	1/25.31	1975ng/ml
6th dilution	1/37.97	1317 ng/ml
7th dilution	1/56.95	878 ng/ml
8th dilution	1/85.43	585 ng/ml

Allow 2 wells per dilution, i.e. a minimal volume of 300 µl (100 µl per well).

Prepare dilutions of the unknown samples in PBS. Please use precision material for this step, especially if the samples must be highly diluted.
The dilution factor for the samples will depend on the approximate lactoferrin starting concentration. Indeed, it is crucial that the optical densities obtained for the unknown samples be located between the lowest value of the standard curve (585ng/ml) and the highest value (10000ng/ml).
For optimal precision, you must ensure that the optical densities obtained for the unknown samples are located near the point of inflexion of the standard curve.
In order to determine approximately how to dilute the unknown samples, you may use the values indicated in the table below, corresponding to mean physiological values. Some pathological samples can vary greatly.

	Dilution factor
Bovine milk:	1/200
Bovine whey:	1/100 – 1/200
Bovine colostrum:	1/200
Bovine blood serum:	1/5

4- Distribute the eight dilutions used for the calibration curve across the plate in duplicate series at the rate of 100 µl per well. Distribute the dilute samples across the plate in duplicate series (100 µl/well).
5- Dilute de conjugate 1:50 with dilution buffer (for example, for one plate dilute 250 µl of the conjugate stock solution in 12,25 ml of diluent).
6- Add 100 µl of the diluted conjugate to each well. Avoid touching the samples in the wells during this operation.
7- Cover the plate with a lid and incubate at 21°C +/- 3°C for one hour.
8- Rinse the plate with the washing solution prepared as instructed in the section “Composition of the Kit”. To do this, dispose of the microplate’s contents by flipping it sharply over a container filled with an inactivating agent. Let the microplate drain upside-down on a sheet of clean absorbent paper so as to eliminate all liquid. Add 300 µl of the washing solution, and then empty the plate once again by flipping it over above the containment vessel. Repeat the entire operation two more times, taking care to avoid the formation of bubbles in the microwells. After the plate has been washed three times proceed to the next step.
Using a plate washer (wether automatic or manual) is also recommended. However, the depth of the needles’ immersion must be set so as not to disturb the layer of reagents adsorbed to the bottom of each well.

9- Add 100 µl of the chromogen solution to each well of the plate. The chromogen solution must be absolutely colourless when it is pipetted into the wells. If a blue colour is visible, this means that the solution has been contaminated.
10- Incubate for 10 minutes at 21°C +/- 3°C. and away from light. This time is given as a guideline only, for in some circumstances it may be useful to lengthen or shorten the incubation time.
11- Add 50 µl of stop solution per microwell. The blue colour will change into a yellow colour.
12- Read the optical densities in the microwells using a plate reader and a 450 nm filter. Results must be read fairly soon after the stopping solution has been added since the chromogen may crystallize in wells with strong signals and distort the results accordingly.

VII – CALCULATING THE RESULTS

In order to calculate the concentrations of lactoferrrin in the unknown samples, it is preferable to use a computer program with Log/Logit or 4-parameter curve fitting options. If such a program is available, key in the eight lactoferrin concentrations in the standard curve (two values per dilution). Name each sample and indicate its dilution factor. The program will determine the four parameters of the standard curve with its correlation coefficient. Interpolate the values in order to obtain the concentrations of the unknown samples.
If it is not possible to make use of a program, the lactoferrin concentrations can be determined from a graph. To do this, use the graph provided in the kit. The bold vertical lines correspond to the concentrations of the standard curve (585ng/ml –878 ng/ml – 1317 ng/ml - 1975 ng/ml - 2963 ng/ml - 4444 ng/ml - 6667 ng/ml - 10000 ng/ml). Plot the optical densities of each point of the calibration curve on the vertical lines and calculate the mean of the optical densities recorded for each point of the curve. Draw a curve that passes as best possible between the eight points of the standard curve. Place the values obtained on the ordinate and draw a horizontal line. From the point of intersection of this horizontal line with the curve, draw a vertical line and read the value in ng/ml on the abscissa. This value must be multiplied by the dilution factor of the sample in order to obtain its lactoferrin concentration (see the example included in the kit).

VIII – ORDERING INFORMATION

QuantELISA Bovine lactoferrin	2x40 tests	BIO K 158/2