# PLUS LACTOFERRIN ELISA

*5091LFER[7]11.20*

## A competitive enzyme immunoassay for screening and quantitative analysis of Lactoferrin in various matrices

**EUROPROXIMA PLUS LACTOFERRIN ELISA**

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## BRIEF INFORMATION

The Plus lactoferrin ELISA is a competitive enzyme immunoassay for measurement of the concentration of lactoferrin. With this ELISA-kit 96 analyses can be performed. Samples and standards are measured in duplicate which means that a total of 40 samples can be analyzed.
The ELISA kit contains all reagents to perform the assay. Reagents for sample preparation are not included in the kit.

## 1. INTRODUCTION

Lactoferrin (LF) is an 80 kDa glycoprotein of the transferrin family capable of binding and transferring iron (Fe3+ ions). It is expressed in most biological fluids and is a major component of the mammalian innate immune system. Its protective effects range from direct antimicrobial activities against a large panel of microorganisms, including bacteria, viruses, fungi and parasites, to anti-inflammatory and anticancer activities. This wide range of activities is due by mechanisms of action utilising not only the capacity of LF to bind iron but also interactions of the glycoprotein with molecular and cellular components of both host and pathogens.
LF is produced by mucosal epithelial cells in various mammalian species and is found in mucosal secretions, including tears, saliva, vaginal fluids, semen, nasal and bronchial secretions, bile, gastrointestinal fluids, urine and most highly in milk, making it the second most abundant protein in milk, after caseins. The concentration of LF in milk varies from 7 g/l in colostrum to 1 g/l in mature milk. LF can also be found in bodily fluids such as blood plasma and amniotic fluid. Furthermore, LF is found in considerable amounts in secondary neutrophil granules, where it plays a significant physiological role.

## 2. PRINCIPLE OF THE PLUS LACTOFERRIN ELISA

The microtiter plate based lactoferrin ELISA consists of one precoated plate (12 strips, 8 wells each). Antibody, horseradish peroxidase (-HRP) labeled lactoferrin and standard solution or sample are added to wells. Free lactoferrin from the samples or standards and lactoferrin-HRP conjugate compete for the specific antibody binding sites (competitive enzyme immunoassay).
After an incubation step of 1 hour the non-bound reagents are removed in a washing step. The amount of bound lactoferrin-HRP conjugate is visualized by the addition of a substrate/chromogen solution ($H_2O_2$/TMB). Bound lactoferrin-HRP conjugate transforms the colourless chromogen into a coloured product.
The substrate reaction is stopped by the addition of sulfuric acid. The colour intensity is measured photometrically at 450 nm. The optical density is inversely proportional to the lactoferrin concentration in the sample.

## 3. SPECIFICITY AND SENSITIVITY

The Limit of detection (LOD) and detection capability (CCβ) are determined under optimal conditions. Cut-off criteria need critical consideration.

| Matrix | Sample preparation | LOD [µg/ml] | CCβ [µg/ml] |
|---|---|---|---|
| UHT milk | 8.1 | 5 | 10 |
| Baby and infant milk powder | 8.2 | -* | 125 |

* The measured concentration of lactoferrin in all blank samples was 0 µg/ml so the LOD was not calculated.

Raw and pasteurized bovine milk contain lactoferrin, therefore CCβ could not be established in these matrices experimentally. The theoretical CCβ is 10 µg/ml (lowest calibration standard × dilution factor) in these matrices.

| Matrix | Sample preparation | Measured lactoferrin concentration [µg/ml] |
|---|---|---|
| Raw milk | 8.1 | 200 |
| Pasteurized milk | 8.1 | 80 |

If the sample is found to be non-compliant, the results shall be verified by re-analysis of the sample using a confirmatory method.

## 4. HANDLING AND STORAGE

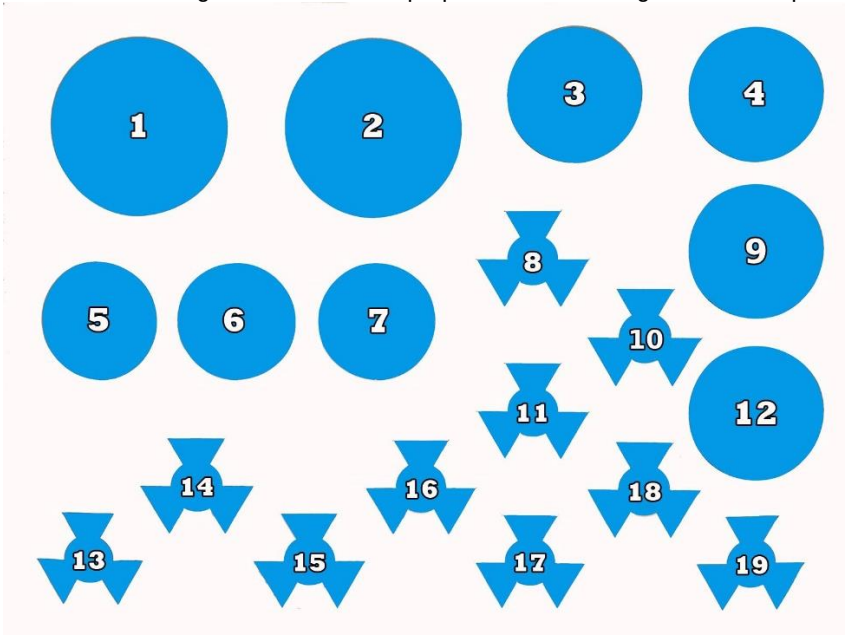
- Kit and kit components should be stored at 2°C to 8°C in a dark place.
- After the expiry date of the kit and/or components has passed, no further quality guarantee is valid.
- Bring all kit components including the microtiter plate to ambient (room) temperature before use.
- Dilute the kit components immediately before use, but after the components are brought to ambient temperature.
- Avoid condensation in the wells of the plate. Bring the sealed plate to ambient temperature before opening the plate sealing.
- The substrate chromogen solution can be stored in a refrigerator (2ºC to 8ºC) until the expiry date stated on the label.
- Exposure of the chromogen solution to light should be avoided.

Degeneration of the reagents may have occurred when the following phenomena are observed:
- A blue colouring of the chromogen solution before transferring it into the wells.
- A weak or no colour reaction in the zero standard wells (E450nm < 0.8).

## 5. KIT CONTENTS

Manual
One sealed (96-wells) microtiter plate (12 strips, 8 wells each), coated with antibody. Plate is ready-to-use.

Position of the reagents in the kit. For preparation of the reagents see Chapter 9.




1. **Dilution buffer** (30 ml, 10x concentrated)
2. **Rinsing buffer** (30 ml, 20x concentrated)
3. **Substrate solution** (12 ml, ready-to-use)
4. **Stop solution** (15 ml, ready-to-use)
5. **Standard** (2 µg/ml lyophilized)
6. **Standard** (2 µg/ml lyophilized)
7. **Standard** (2 µg/ml lyophilized)
8. **Conjugate solution** (100 µl; 100x concentrated)
9. not in use
10. **Antibody solution** (100 µl; 100x concentrated)
11. not in use
12. not in use
13. not in use
14. not in use
15. not in use
16. not in use
17. not in use
18. not in use
19. not in use

## 6. EQUIPMENT AND MATERIALS REQUIRED BUT NOT PROVIDED

- Scales and weighing vessels
- Gloves
- Homogeniser (vortex, mixer)
- Automated microtiter plate washer or 8-channel micropipette 100 – 300 µl
- Microtiter plate shaker
- Microtiter plate reader with 450 nm filter
- Micropipettes, 100 – 1000 µl
- Multipipette with 2.5 ml combitips
- Methanol 100%
- Distilled water

## 7. PRECAUTIONS

- This kit may contain hazardous substances. For hazard notes please refer to the appropriate safety data sheets (SDS).
- Avoid contact of all biological materials with skin and mucous membranes.
- Do not pipette by mouth.
- Do not eat, drink, smoke, store or prepare foods, or apply cosmetics within the designated work area.
- Do not use components past expiration date and do not use components from different lots.
- Each well is ultimately used as an optical cuvette. Therefore, do not touch the under surface of the wells, prevent damage and dirt.
- All components should be completely dissolved before use. Take special attention to the substrate and rinsing buffer, which crystallize at +4ºC.
- Optimal results will be obtained by strict adherence to this protocol. Careful pipetting and washing throughout this procedure are necessary to maintain good precision and accuracy.

## 8. SAMPLE PREPARATION

8.1 Milk

- Vortex the milk sample for 30 seconds.
- First, dilute the sample 1 : 1 with 100% methanol, i.e. 200 µl milk + 200 µl 100% methanol.
- Vortex the sample for 30 seconds.
- Then, further dilute the sample 1 : 100 with dilution buffer, i.e. 10 µl of the 1 : 1 diluted milk in methanol + 990 µl dilution buffer.
- Vortex the sample for 30 seconds.
- Use 50 µl of the diluted milk sample in the ELISA.

8.2 Baby and infant milk powder

- Weigh out 0.5 gram baby milk powder into a tube.
- Add 12 ml distilled (or pure) water, with a temperature of 4°C.
- Mix 5 minutes head over head, or mix during the 5 minutes a few times manually.
- Store the dissolved milk sample for 15 minutes at 4°C.
- Dilute 10 ul of this milk solution further with 990 ul dilution buffer (delivered with the kit).
- Vortex the sample for 30 seconds.
- Use 50 µl of the diluted milk samples in the ELISA

This sample preparation is optimal for infant formula, containing in average 300 to 500 µg/g (ppm). The dilution can be customized when the concentration of lactoferrin is outside this range.

## 9. PREPARATION OF REAGENTS

Before beginning the test, the reagents should be brought up to ambient temperature. Any reagents not used should be put back into storage immediately at +2ºC to +8ºC. Prepare reagents fresh before use.

Microtiter plate
Return unused strips into the resealable bag with desiccant and store at +2ºC to +8ºC for use in subsequent assays. Retain also the strip holder.

Dilution buffer
This buffer is used for the dilution of conjugate, antibody and samples. The dilution buffer is 10x concentrated. Dilute the buffer 1:10 (10 ml buffer + 90 ml distilled water) before use. The concentrated buffer should be at room temperature (20ºC to 25ºC) and thoroughly mixed. Concentrated buffer can show precipitates, mix well before dilution.

Standard
Prepare a dilution range of lactoferrin standards. Add 2 ml of dilution buffer to the lactoferrin standard and mix. This solution contains 2 µg lactoferrin per ml. Pipette 0.25 ml of this solution into a clean tube and add 0.25 ml of dilution buffer. Continue to make a dilution range of 2.0, 1.0, 0.5, 0.2, 0.1 and 0.05 µg/ml.
For prolonged storage: freeze aliquots at -20ºC.

Conjugate
The conjugate is delivered 100x concentrated. Spin down the conjugate in the vial by a short centrifugation step (1 min., 1000 x g). Add 5 µl of the concentrated conjugate solution to 495 µl dilution buffer. Per 2 x 8 wells 400 µl is required. Store unused concentrated conjugate at 2°C - 8°C.

Antibody
The antibody is 100x concentrated. Spin down the antibody in the vial by a short centrifugation (1 min. 1000 x g). Add 5 µl of the concentrated antibody to 495 µl dilution buffer. Per 2 x 8 wells 400 µl of antibody solution is required. Store concentrated antibody immediately upon use at 2°C - 8°C.

Rinsing buffer
The rinsing buffer is delivered 20x concentrated. Prepare dilutions freshly before use. For each strip 20 ml of diluted rinsing buffer is used (1 ml concentrated rinsing buffer + 19 ml distilled water).

Substrate/chromogen solution
The substrate/chromogen solution (ready-to-use) tends to precipitate at +4ºC. Take care that this vial is at room temperature when used (keep in the dark) and mix the content before pipetting into the wells.

## 10. ASSAY PROCEDURE

Rinsing protocol

In ELISA's, between each immunological incubation step, unbound components have to be removed efficiently. This is reached by appropriate rinsing. It should be clear that each rinsing procedure must be carried out with care to guarantee good inter- and intra-assay results.
Basically, manual rinsing or rinsing with automatic plate wash equipment can be performed as follows:

Manual rinsing

1. Empty the contents of each well by turning the microtiter plate upside down and remove residual liquid by striking the plate against a paper towel.
2. Fill all the wells to the rims (300 µl) with rinsing solution.
3. This rinsing cycle (1 and 2) should be carried out 3 times.
4. Turn the plate upside down and empty the wells by a firm short vertical movement.
5. Place the inverted plate on absorbent paper towels and tap the plate firmly to remove residual rinsing solution from the wells.
6. Take care that none of the wells dry out before the next reagent is dispensed.

Rinsing with automatic microtiter plate wash equipment

When using automatic plate wash equipment, check that all wells can be aspirated completely, that the rinsing solution is nicely dispensed reaching the rim of each well during each rinsing cycle. The washer should be programmed to execute three rinsing cycles.

### Assay Protocol

1. Prepare samples according to Chapter 8 and prepare reagents according to Chapter 9.

2. Pipette 100 µl of dilution buffer in duplicate (wells H1, H2, blank).
   Pipette 50 µl of dilution buffer (zero standard, Bmax) in duplicate (wells A1, A2).
   Pipette 50 µl of each of the standard solutions in duplicate (wells B1,2 to G1,2 i.e. 0.05, 0.1, 0.2, 0.5, 1.0 and 2.0 µg/ml).

3. Pipette 50 µl of each sample solution in duplicate into the remaining wells of the microtiter plate.

4. Pipette 25 µl of conjugate (lactoferrin-HRP) to all wells, except H1 and H2.

5. Pipette 25 µl of antibody solution to all wells except H1 and H2.

6. Seal the microtiter plate and shake the plate for a few seconds on a microtiter plate shaker.

7. Incubate for 1 hour in the dark at room temperature (20ºC to 25ºC).

8. Discard the solution from the microtiter plate and wash 3 times with rinsing buffer.

9. Pipette 100 µl of substrate solution into each well.

10. Incubate 30 minutes in the dark at room temperature (20ºC to 25ºC).

11. Add 100 µl of stop solution to each well.

12. Read the absorbance values immediately at 450 nm.

## 11. INTERPRETATION OF RESULTS

Subtract the mean optical density (O.D.) of the wells H1 and H2 (Blank) from the individual O.D. of the wells containing the standards and the samples.

The O.D. values of the six standards and the samples (mean values of the duplicates) are divided by the mean O.D. value of the zero standard/ Bmax (wells A1 and A2) and multiplied by 100. The zero standard/ Bmax is thus made equal to 100% (maximal absorbance) and the other O.D. values are quoted in percentages of the maximal absorbance.

$$\frac{\text{O.D. standard (or sample)}}{\text{O.D. zero standard/ Bmax}} \times 100 = \text{percentage maximal absorbance}$$

Calibration curve:
The values (% maximal absorbance) calculated for the standards are plotted on the Y-axis versus the analyte equivalent concentration (µg/ml) on a logarithmic X-axis.

Alternative for calibration curve:
The value of absorption (logit) calculation of the standards are plotted on Y-axis versus the analyte equivalent concentration on a logarithmic X-axis.

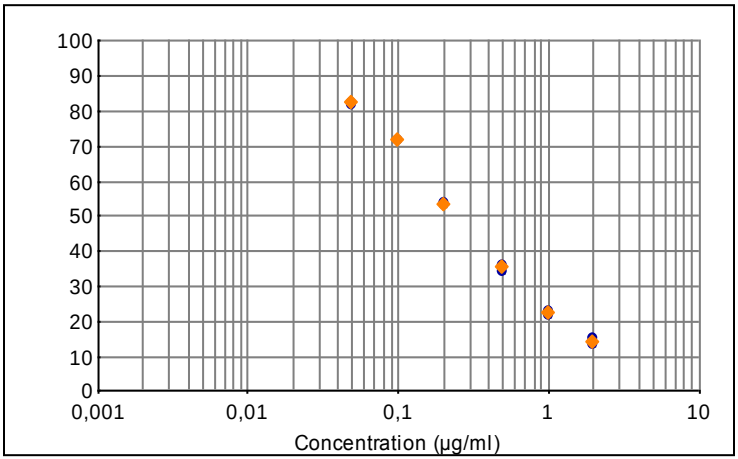



**Figure 1: Example of a calibration curve**

The amount of lactoferrin in the samples is expressed as lactoferrin equivalents. The lactoferrin equivalents in the samples (µg/ml) corresponding to the % maximal absorbance of each extract can be read from the calibration curve.

8.1 Milk

To obtain the lactoferrin content in the samples, the calculated lactoferrin concentration has to be multiplied by a factor 200.

8.2 Baby and infant milk powder

To obtain the lactoferrin content in the samples µg/g, the calculated lactoferrin concentration has to be multiplied by a factor 2500.

## 12. LITERATURE

González-Chávez, S.A., Arévalo-Gallegos, S., and Rascón-Cruz, Q. (2009). Lactoferrin: structure, function and applications (review). Int. J. Antimicrobial Agents, **33**, 301.e1-301.e8.

Adlerova, L., Bartoskova, A., and Faldyna, M. (2008). Lactoferrin: a review. Veterinarni Medicina **53 (9)**, 457-468.

EFSA: Scientific opinion on bovine lactoferrin. EFSA Journal (2012), **10(5)**, 2701.

Farnaud, S. and Evans, R.W. (2003). Lactoferrin- a multifunctional protein with antimicrobial properties. Molecular Immunol. **40**, 395-405.

## 13. ORDERING INFORMATION

For ordering the Plus lactoferrin ELISA kit, please use cat.code 5091LFER.

## 14. REVISION HISTORY

"Plus" has been added to the already existing name of the manual. The kit and the kit ordering code will remain unchanged.

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