PLUS LACTOFERRIN FAST ELISA

5091LFERF[6]11.20

A competitive enzyme immunoassay for screening and quantitative analysis of Lactoferrin in infant milk powder

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BRIEF INFORMATION

The EuroProxima Plus lactoferrin FAST ELISA is a competitive enzyme immunoassay for the screening and quantitative detection of lactoferrin in infant milk powder. 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 analysed.
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.
Lactoferrin is found in mucosal secretions, urine and plasma. The highest concentration of human lactoferrin is found in milk and colostrum. In bovine milk the lactoferrin concentration varies from 0.05 to 0.5 g/L.
After the European Food Safety Authority approved bovine lactoferrin as novel food ingredient in 2012, the demand for this substance has exploded due to the application in infant powder milk, especially in China.
To monitor the concentration of added bovine lactoferrin, R-Biopharm Nederland presents the Plus Lactoferrin FAST ELISA. This ELISA test is specifically developed for the use in infant milk powder.

2. PRINCIPLE OF THE PLUS LACTOFERRIN ELISA

The microtiter plate based Plus Lactoferrin FAST ELISA consists of one precoated plate with antibody against lactoferrin (12 strips, 8 wells each). 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 30 minutes at room temperature, 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 the detection capability (CCβ) are determined under optimal conditions. Cut-off values need critical consideration.

Matrix	Sample preparation	LOD [µg/g]	CCβ [µg/g]
Infant milk powder	8.1	103	200

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.

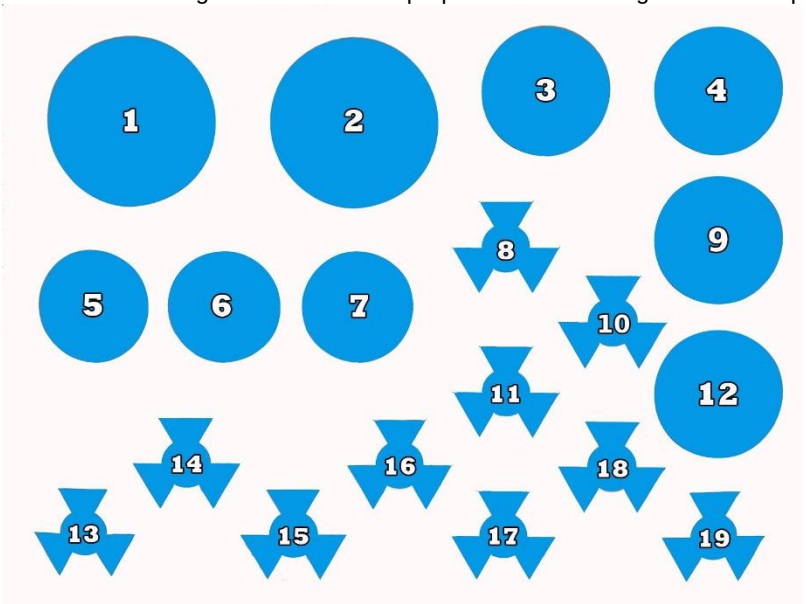
Degeneration of the reagents may have occurred when the following phenomena are observed:
- A blue colouring of the chromogen solution before transferring it into the wells.
- A weak or no colour reaction in the zero standard wells (E450nm < 0.8).

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** (2.5 µg/ml lyophilized)
6. **Standard** (2.5 µg/ml lyophilized)
7. **Standard** (2.5 µg/ml lyophilized)
8. **Conjugate solution** (150 µl; 100x concentrated)
9. not in use
10. not in use
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, 10 – 1000 µl
- Multipipette with 2.5 ml combitips
- 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 Infant milk powder

- Weigh 1 g of sample and fill up to 10 ml with water
- Vortex and then mix end-to-end for 5 min
- Dilute the sample 200× in dilution buffer in a following way:
  add 50 µl of extracted sample to 450 µl of the dilution buffer (10× dilution), vortex and then add 50 µl of 10× dilution to 950 µl of the dilution buffer and vortex to obtain 200× dilution
- Use 50 µl per well in the ELISA test

8.2 Infant milk powder containing > 2500 µg/g of lactoferrin

Sample preparation for infant milk powder samples requiring further dilutions to obtain readings within the linear part of the calibration range.

- Prepare a sample containing a high level of lactoferrin as in point 8.1
- Prepare a blank infant milk powder sample (sample containing no lactoferrin) as in point 8.1
- Use blank infant milk powder extract to dilute the extracted sample containing high level of lactoferrin, for example: for a final dilution factor of 1/10000 mix 100 µl of the extracted sample with 400 µl of blank infant milk powder extract.

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 serial dilution of lactoferrin standard. Add 2 ml of dilution buffer to the lactoferrin standard and mix. This solution contains 2.5 µg of lactoferrin per ml. Pipette 0.25 ml of this solution into a clean tube and add 0.25 ml of dilution buffer. Continue to obtain a serial dilution range of 2.5, 1.25, 0.625, 0.313, 0.156 and 0.078 µg/ml.

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 800 µl is required. Store unused concentrated conjugate 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

Unbound components have to be removed efficiently between each incubation step in ELISAs. This is achieved by appropriate rinsing. Each rinsing procedure must be carried out with care to guarantee good inter- and intra-assay results.
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 in duplicate (wells A1, A2, maximal signal).
   Pipette 50 µl of each of the standard solutions in duplicate (wells B1,2 to G1,2 i.e. 0.078, 0.156, 0.313, 0.625, 1.25 and 2.5 µg/ml).

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

4. Pipette 50 µl of conjugate (lactoferrin-HRP) to all wells, except H1 and H2.

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

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

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

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

9. Incubate 15 minutes in the dark at room temperature (20ºC to 25ºC).

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

11. 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.

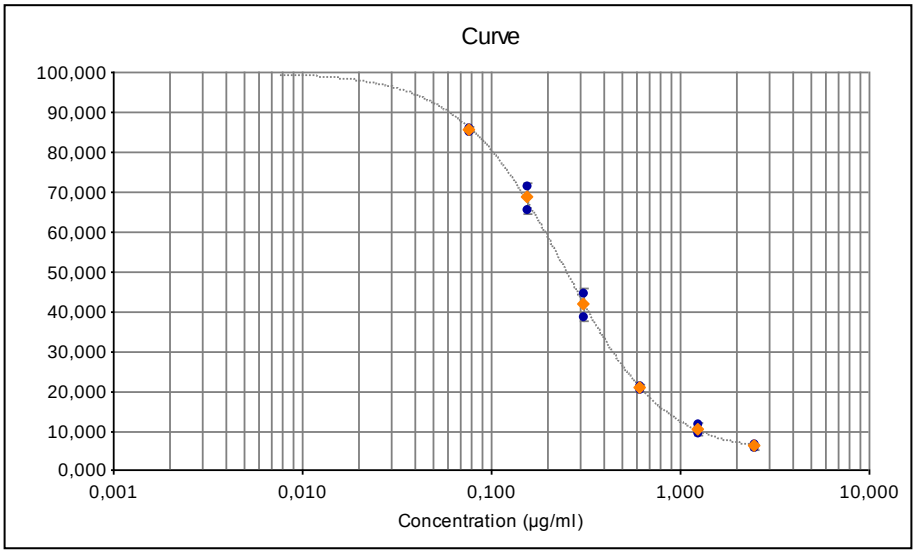


Figure 1: Example of a calibration curve

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 Infant milk powder

To obtain the lactoferrin content in the samples in µg/g, the calculated lactoferrin concentration has to be multiplied by a factor 2000.

12. LITERATURE

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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 EuroProxima Plus Lactoferrin FAST ELISA kit, please use catalogue code 5091LFERF.

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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