PLUS AFLATOXIN M1 FAST ELISA

5121AFMF[5]11.20

A competitive enzyme immunoassay for quantitative analysis of Aflatoxin M1 in milk and milk products

EUROPROXIMA PLUS AFLATOXIN M1 FAST ELISA

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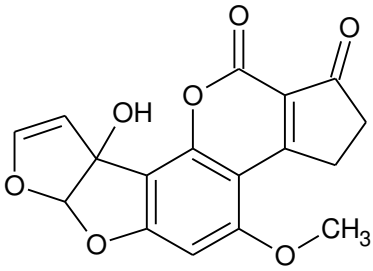
BRIEF INFORMATION

The Plus aflatoxin M1 fast ELISA is a competitive enzyme immunoassay for the screening of samples of milk and milk products. The test is based on antibodies directed against aflatoxin M1. The ELISA kit contains a 96 well microtiter plate as well as all essential reagents including ready-to-use standards to perform the test. Methods for a fast and efficient extraction of aflatoxin M1 from different matrices are included in the kit manual.

1. INTRODUCTION



Chemical structure of aflatoxin M1

Aflatoxins are highly toxic mycotoxins. They are produced by some *Aspergillus* moulds, i.e. *Aspergillus flavus, A. paraciticus* and the rare *A. nomius*. Aflatoxin M1 is the hydroxylated metabolite of aflatoxin B1 and can be found in milk obtained from livestock that have ingested contaminated feed. The main sources of aflatoxins in feeds are peanut meal, maize and cottonseed meal.
Also human breast milk can contain aflatoxin M1 after the lactating woman has consumed food contaminated with aflatoxin B1.

For Aflatoxin M1 Maximum tolerance Levels (ML) are established legally in Europe.
These limits are as follows:

Milk, including raw milk, milk for the production of milk based products and heat treated milk: 50 ppt

Infant formulae and follow-on formulae, including infant milk and follow-on milk: 25 ppt

Dietary foods for special medical purposes intended specifically for infants: 25 ppt.

2. PRINCIPLE OF THE PLUS AFLATOXIN M1 FAST ELISA

The kit is based on a microtiter plate (12 strips, 8 wells each), precoated with aflatoxin M1 antibodies.
Standards solution or sample are added to the wells. Aflatoxin M1 from the samples or standards is bound by the specific antibody binding sites.
After an incubation step of 30 minutes, the non-bound reagents are removed in a washing step. Horseradish peroxidase (HRP) labeled aflatoxin M1 is added to the wells.
After an incubation step of 15 minutes, the non-bound conjugate is removed in a washing step. The amount of bound aflatoxin M1-HRP conjugate is visualized by the addition of a substrate/chromogen solution (H_2O_2/TMB). Bound aflatoxin M1-HRP conjugate transforms the colourless chromogen into a colored product.
The substrate reaction is stopped by the addition of sulfuric acid. The colour intensity is measured photo metrically at 450 nm. The optical density is inversely proportional to the aflatoxin M1 concentration in the sample.

3. SPECIFICITY AND SENSITIVITY

The Plus Aflatoxin M1 fast ELISA utilizes antibodies raised in mouse against protein conjugated Aflatoxin M1.
Cross- reactivity: Aflatoxin M1 100%
Aflatoxin M2 70%

The cross-reactivities are determined in a buffer system. The reported values may be different in samples due to matrix effects.
The test cannot discriminate between analytes and cross-reactive substances.

The Limit of detection (LOD) is determined under optimal conditions. Cut-off criteria need critical consideration.

Matrix	Procedure	LOD (ppt)
Milk	8.1	5
Cheese	8.2	<10
Butter	8.3	<10

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. For repeated use store kit components as specified under chapter 9.
- 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:

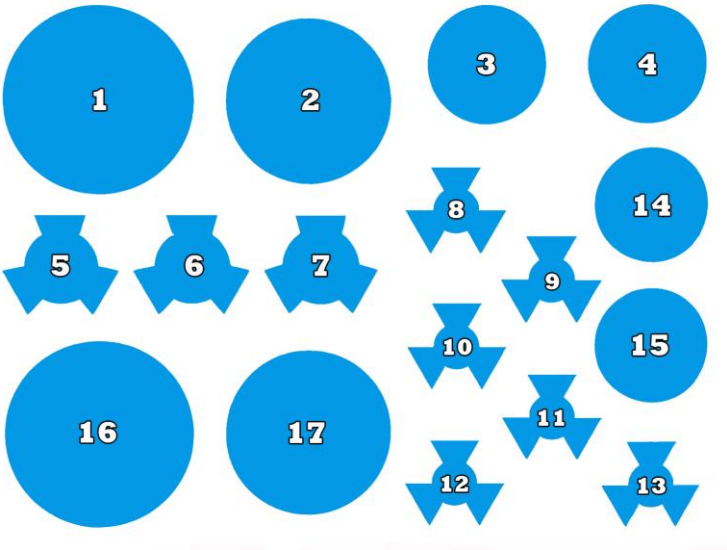
- A blue colouring of the chromogen solution before transferring it into the wells.
- A weak or no colour reaction in the zero standard wells (E450nm < 0.8).

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. **Sample dilution buffer** (40 ml, Ready-to-use)
2. **Rinsing buffer** (30 ml, 20x concentrated)
3. **Substrate solution** (12 ml, Ready-to-use)
4. **Stop solution** (15 ml, Ready-to-use)
5. **Conjugate Solution** (150 µl, 100 times concentrated)
6. **Standard solution** (1000 pg/ml, Ready-to-use))
7. **Zero standard solution** (4ml, Ready-to-use)
8 **Standard solution 1** (2ml, Ready-to-use) **6.25 pg/ml**
9. **Standard solution 2** (2ml, Ready-to-use) **12.50 pg/ml**
10. **Standard solution 3** (2ml, Ready-to-use) **25 pg/ml**
11 **Standard solution 4** (2ml, Ready-to-use) **50 pg/ml**
12. **Standard solution 5** (2ml, Ready-to-use) **100 pg/ml**
13 **Standard solution 6** (2ml, Ready-to-use) **200 pg/ml**
14. **Dilution buffer** (15 ml, Ready-to-use)
15. not in use
16. not in use
17. not in use

6. EQUIPMENT AND MATERIALS REQUIRED BUT NOT PROVIDED

- Scales and weighing vessels
- Gloves
- Fume hood
- Homogeniser (vortex, mixer)
- Centrifuge (2000 x g)
- Automated microtiter plate washer or 8-channel micropipette 100 – 300 µl
- Microtiter plate shaker
- Microtiter plate reader with 450 nm filter
- Glass test tubes (10 – 15 ml)
- Micropipettes, 100 – 1000 µl
- Multipipette with 2.5 ml combitips
- Folded filtration paper, 90 mm
- Aluminium foil or parafilm
- Dichloromethane
- Heptane
- Distilled water
- 0.1 M HCl

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 PREPARATIONS

8.1 Milk samples

Samples have to be defatted: Centrifuge cold milk samples for 10 minutes, 2000 x g, 4°C. Remove the upper fat layer using a spatula. Use 100 µl portions of the defatted milk samples in the ELISA test.

8.1.1 Milk powder samples

Reconstitute the milk powder according to manufacturers instruction. If the manufacturers instruction is not available, than pipette 10 ml of deionized or distilled water to 1 g of milk powder. Mix well and defat the reconstituted samples: Centrifuge cold reconstituted milk samples for 10 minutes, 2000 x g, 4°C. Remove the upper fat layer using a spatula. Use 100 µl portions of the defatted samples in the ELISA test.

8.2 Cheese samples

- Weigh 2 g of finely cut and subsequently homogenised cheese in the 15 ml tube.
- Add 4 ml of 0.1 M HCl.
- Add 8 ml of dichloromethane.
- Mix 15 minutes head over head, centrifuge (5 minutes, 2000 x g) at 20°C - 25°C and remove the upper layer (HCl).
- Filter the cheese extract through a 90 mm filter.
- Take 4 ml of the filtrate and evaporate to dryness at 50ºC, under a mild stream of nitrogen.
- Dissolve the residue in 1 ml of sample dilution buffer.
- Add 1 ml of heptane (for defatting) and vortex.
- Centrifuge (5 minutes, 2000 x g).
- Pipette 100 µl of the layer underneath to test in the wells of the ELISA plate.

8.3 Butter samples

- Weigh 2 g of finely cut and subsequently homogenised butter in the 15 ml tube.
- Add 4 ml of 0.1 M HCl.
- Add 8 ml of dichloromethane.
- Mix 15 minutes head over head, centrifuge (5 minutes, 2000 x g) at 20°C - 25°C and remove the upper layer (HCl).
- Filter the butter extract through a 90 mm filter.
- Take 4 ml of the filtrate and evaporate to dryness at 50ºC, under a mild stream of nitrogen.
- Dissolve the residue in 1 ml of sample dilution buffer.
- Add 1 ml of heptane (for defatting) and vortex.
- Centrifuge (5 minutes, 2000 x g).
- Pipette the layer underneath into a clean glass tube.
- Add 1 ml heptane and vortex.
- Centrifuge (5 minutes, 2000 x g).
- Pipette 100 µl of the layer underneath and transfer this 100 µl to the wells of the ELISA plate.

9. PREPARATION OF REAGENTS

Before starting the test, the reagents should be brought up to ambient temperature. Any reagents not used should be put back into storage immediately at 2°C to 8°C.

Microtiter plate
Return unused strips into the plastic zip lock bag with desiccant and store at 2°C to 8°C for use in subsequent assays. Retain also the strip holder.

Sample Dilution buffer
This buffer is used to dissolve the evaporated sample residues. This buffer can become jelly. Allow the buffer to adapt to ambient temperature and mix vigorously until a homogeneous solution is obtained.

Dilution buffer
This is the dilution buffer for the dilution of the conjugate.

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).

Conjugate solution
Prepare reagents freshly before use
The conjugate (Aflatoxin M1-HRP) is delivered 100x concentrated. Spin down the conjugate in the vial by a short centrifugation step (1 minute, 1000 x g). Add 20 µl of the concentrated conjugate solution to 2 ml of dilution buffer. Per 2 x 8 wells 1600 µl of diluted conjugate is required. Store unused concentrated conjugate at 2°C to 8°C.

Substrate/chromogen solution
The substrate/chromogen solution (ready-to-use) tends to precipitate at 4°C.
Take care that this vial is at room temperature (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 done 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 washing solution in 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 the zero standard in duplicate (wells H1, H2, blank).
   Pipette 100 µl of the zero standard in duplicate (wells A1, A2, maximal signal).
   Pipette 100 µl of each of the Aflatoxin M1 standard solutions in duplicate (wells B1,2 to G1,2 i.e. 6.25, 12.50, 25, 50, 100 and 200 pg/ml).

3. Pipette 100 µl of each sample solution in duplicate into the remaining wells of the microtiter plate.

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

5. Incubate for 30 minutes in the dark at 20°C to 25°C.

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

7. Pipette 100 µl of conjugate (Aflatoxine M1-HRP) to all wells, except wells H1 and H2.

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

9. Incubate for 15 minutes in the dark at 20°C to 25°C.

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

11. Pipette 100 µl of substrate solution into each well. Incubate 15 minutes at 20°C to 25°C.

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

13. 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 (percentage maximal absorbance) calculated for the standards are plotted on the Y-axis versus the analyte equivalent concentration (pg/ml) on a logarithmic X-axis.

Alternative for calibration curve:
The absorbtion value of the standards is plotted on the Y-axis versus the concentration on the X-axis. The Y-axis is in logit the Y-axis is logarithmic.

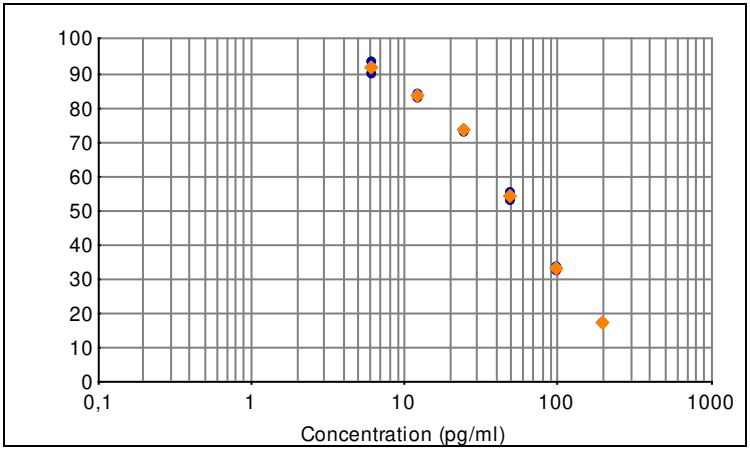


Figure 1 : Example of a calibration curve

The amount of Aflatoxin M1 in the samples is expressed as Aflatoxin M1 equivalents. The Aflatoxin M1 equivalents in the samples (pg/ml) corresponding to the percentage maximal absorbance of each extract can be read from the calibration curve.

8.1 Milk samples
The concentration of Aflatoxin M1 in milk samples can directly be read from the standard curve.

8.1.1 Milk powder samples
The ML of Aflatoxin M1 for infant formulae and follow-on formulae refers to the product ready-to-use (marketed as such or after reconstitution as instructed by the manufacturer). So, the concentration of Aflatoxin M1 in these reconstituted samples can directly be read from the standard curve.

8.2 Cheese samples
The concentration of Aflatoxin M1 in cheese samples can directly be read from the standard curve.

8.3 Butter samples
The concentration of Aflatoxin M1 in butter samples can directly be read from the standard curve.

12. LITERATURE

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13. ORDERING INFORMATION

For ordering the Plus Aflatoxin M1 fast ELISA kit, use cat. code 5121AFMF.

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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