

AFLATOXIN B1 Sensitive ELISA

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A competitive enzyme immunoassay for
screening and quantitative analysis of
aflatoxin B1 in various matrices

EUROPROXIMA AFLATOXIN B1 SENSITIVE ELISA

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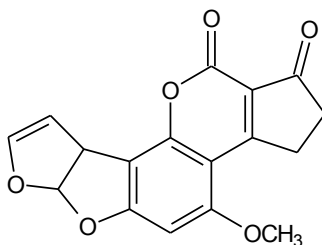
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BRIEF INFORMATION

The aflatoxin B1 ELISA is a competitive enzyme immunoassay for measurement of the concentration of aflatoxin B1. With this ELISA-kit 96 analyses can be performed. Samples and standards are measured in duplicate which means that a total of 41 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



Chemical structure of Aflatoxin B1

Aflatoxins (Aflatoxins B₁, B₂, G₁, G₂) are a group of structurally related, extremely toxic compounds produced by the moulds *Aspergillus flavus*, *A. paraciticus* and *A. nomius*. Aflatoxins cause cancer, mainly of the liver but also of the gut, lungs and breasts. These moulds mainly occur on food, feed and their ingredients derived from tropical and sub-tropical areas. The most pronounced contamination has been encountered in cereals, rice, maize, soy, tree nuts and peanuts.

For aflatoxins maximum levels (MLs) are established legally in Europe. Depending on the products used for animal feed or direct human consumption the MLs vary from 50 to 2 µg/kg (ppb). For baby food, processed cereal-based foods for infants and young children and for dietary foods intended specifically for infants, the ML for aflatoxin B₁ is set at 0.1 µg/kg (0.1 ppb).

2. PRINCIPLE OF THE AFLATOXIN B1 ELISA

The microtiter plate based Aflatoxin B1 ELISA consists of 12 strips, each 8 wells, precoated with anti-aflatoxin B1 antibodies. Standard solution or sample are added to the wells. After an incubation step of 30 minutes, followed by a washing step, horseradish peroxidase (-HRP) labeled aflatoxin B1 is added. After a subsequent incubation step of 30 minutes, followed by a washing step, the amount of bound aflatoxin B1-HRP conjugate is visualized by the addition of a substrate/chromogen solution (H₂O₂/TMB). Bound aflatoxin B1 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 Aflatoxin B1 concentration in the sample.

3. SPECIFICITY AND SENSITIVITY

The aflatoxin B1 ELISA utilizes antibodies raised in mouse against protein conjugated Aflatoxin B1. The reactivity pattern of the antibody is:

Cross-reactivity:	Aflatoxin B1	100%
	Aflatoxin B2	20%
	Aflatoxin G1	17%
	Aflatoxin G2	4%

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 (ppb)
Baby / Infant food	8.1	0.03
Cereals	8.1	0.03
Nuts	8.1	0.05
Liver	8.1	0.05
Red pepper	8.1	0.5
Feed	8.1	2.5
Serum	8.1	0.03

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 (2°C to 8°C) 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:

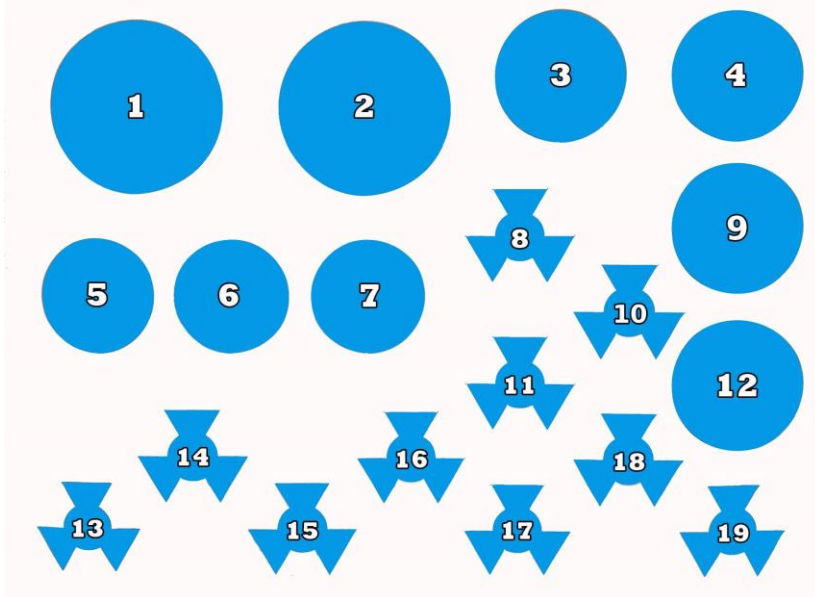
- A blue colouring of the chromogen solution before transferring it into the wells.
- A weak or no colour reaction in the zero standard wells ($E_{450nm} < 0.8$).

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** (20 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. **Standard** (lyophilised, black cap)
6. **Standard** (lyophilised, black cap)
7. **Standard** (lyophilised, black cap)
8. **Conjugate Solution** (150 μ l, 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
- Fume hood
- Evaporation equipment
- 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 filter
- n-Heptane
- Trichloromethane or Dichloromethane
- Methanol
- Disodium hydrogen phosphate (Na_2HPO_4)
- Sodium chloride (NaCl)
- Potassium dihydrogen phosphate (KH_2PO_4)
- 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 $+4^\circ\text{C}$.
- 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.

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8. SAMPLE PREPARATION

8.1 Extraction

- The first step in the sample preparation process depends on the nature of the compound to be tested
- In general a homogenous sample has to be obtained from a representative part of the compound. Alternative techniques such as grinding, pulverizing, etc can be used
- Approximately 50 – 100 gram of sample is ground and pulverised into a fine homogenous compound
- Add 10 ml distilled water, to 2 gram of homogenised ground sample and vortex.
- Add 20 ml of dichloromethane to the mixture
Instead of dichloromethane, trichloromethane can be used
- Shake thoroughly, head over head 5 – 10 minutes
- Centrifuge the sample (10 minutes, 2000 x g)
- Filtrate the layer underneath over a folded filter
- Evaporate 2.5 ml of the filtrate under a mild stream of nitrogen at 50°C
- Dissolve the residue in 1 ml sample/standard dilution buffer (see chapter 9)
- Add 1 ml of n-heptane
- Vortex for 1 minute
- Centrifuge: 10 minutes, 2000 x g
- Pipette 200 µl of the layer underneath into a clean tube
- For **baby food, cereals, nuts and serum** 100 µl of this solution is used in the ELISA
- For **liver** this solution is diluted 1:2 in sample/standard dilution buffer. 100 µl of this dilution is used in the ELISA
- For **red pepper** this solution is diluted 1:20 in sample/standard dilution buffer. 100 µl of this dilution is used in the ELISA.
- For **feed** this solution is diluted 1:100 in sample/standard dilution buffer. 100 µl of this dilution is used in the ELISA.

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 +2°C to +8°C. Prepare reagents fresh before use.

Microtiter plate

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

Dilution buffer

The dilution buffer is used for the preparation of the conjugate and is supplied ready-to-use in the kit.

Phosphate Buffered Saline (PBS)

Na ₂ HPO ₄	0.77 g
KH ₂ PO ₄	0.18 g
NaCl	8.94 g

Fill with distilled water to 1 liter, adjust pH to 7.4 (7.2 – 7.5)

Sample/standard dilution buffer

Prepare fresh before use. 45 ml PBS + 5 ml of 100% methanol.

Standard solutions

Prepare a dilution range of the aflatoxin standard. 3 Vials of lyophilized standard are supplied to prepare fresh standards for each run. Add 2.0 ml of the freshly prepared sample/standard dilution buffer to the vial of aflatoxin B1 standard and mix. This aflatoxin B1 solution contains 100 pg/ml.

Continue to make a dilution range resulting in a concentration range of 50, 25, 12.5 and 6.25 pg/ml in sample/standard dilution buffer.

Standard 0 ppt is sample/standard dilution buffer.

Conjugate solution (150 µl)

The conjugate (aflatoxin B1-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.

Rinsing buffer

The rinsing buffer is delivered 20x concentrated. Prepare dilutions freshly before use. For each strip 20ml 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 +4°C. 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 μ l) 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 μ l of the sample/standard dilution buffer in duplicate (wells G1, G2, blank).
Pipette 100 μ l of the sample/standard dilution buffer (zero standard, Bmax) in duplicate (wells A1, A2).
Pipette 100 μ l of each of the standard solutions in duplicate (wells B1,2 to F1,2 i.e. 6.25, 12.5, 25, 50 and 100 pg/ml).
3. Pipette 100 μ l 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 room temperature (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 (aflatoxin-HRP) to all wells, except G1 and G2.
8. Seal the microtiter plate and shake the plate for a few seconds on a microtiter plate shaker.
9. Incubate for 30 minutes in the dark at room temperature (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.
12. Incubate 30 minutes in the dark at room temperature (20°C to 25°C).
13. Add 100 µl of stop solution to each well.
14. Read the absorbance values immediately at 450 nm.

11. INTERPRETATION OF RESULTS

Subtract the mean optical density (O.D.) of the wells G1 and G2 (Blank) from the individual O.D. of the wells containing the standards and the samples.

The O.D. values of the five 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.

O.D. standard (or sample)

-----x 100 = percentage maximal absorbance

O.D. zero standard/ Bmax

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 value of absorption (logit) calculation of the standards are plotted on Y-axis versus the analyte equivalent concentration on a logarithmic X-axis.

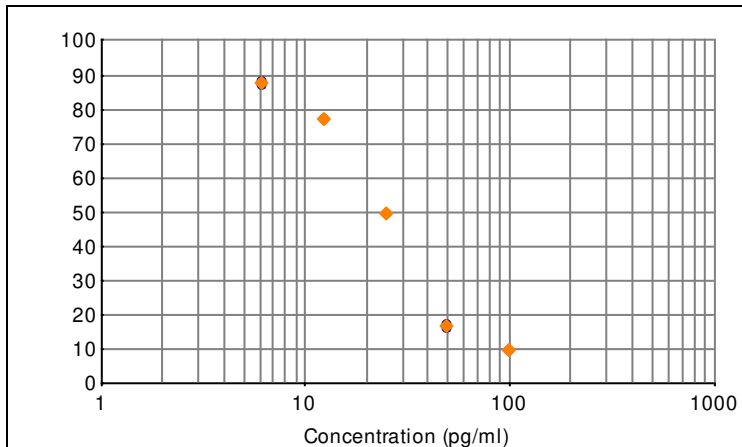


Figure 1 : Example of a calibration curve

The amount of aflatoxin B1 in the samples is expressed as aflatoxin B1 equivalents. The aflatoxin B1 equivalents in the samples (pg/ml) corresponding to the percentage maximal absorbance of each extract can be read from the calibration curve.

Sample calculation factor

Baby food, cereals, nuts and serum

To obtain the aflatoxin content in baby food, cereals, nuts and serum the calculated aflatoxin concentration has to be multiplied by a factor 4

Liver samples

To obtain the aflatoxin content in liver samples the calculated aflatoxin concentration has to be multiplied by a factor 8.

Red pepper samples

To obtain the aflatoxin content in red pepper samples the calculated aflatoxin concentration has to be multiplied by a factor 80.

Feed samples

To obtain the aflatoxin content in feed samples the calculated aflatoxin concentration has to be multiplied by a factor 400.

12. LITERATURE

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Commission Regulation (EC) No 2174/2003 of 12 December 2003 amending Regulation (EC) no 466/2001 as regards aflatoxins. Off. J. European Commun. L326 (2003) 12-15.

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13. ORDERING INFORMATION

For ordering the aflatoxin B1 Sensitive ELISA kit, please use cat. code 5121AFBS.

14. REVISION HISTORY

The manual is adapted to a new layout of the test kit. Several textual changes are added.