AFLATOXIN B1 Sensitive ELISA

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

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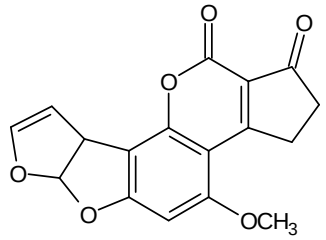
BRIEF INFORMATION

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 B_1, B_2, G_1, G_2) are a group of structurally related, extremely toxic compounds produced by the moulds *Aspergillus flavus, A. paraciticus* and *A. nomius* [1]. 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 [2].
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) [3-6]. For baby food, processed cereal-based foods for infants and young children and for dietary foods intended specifically for infants, the ML for aflatoxin B_1 is set at 0.1 µg/kg (0.1 ppb) [7].

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 (H_2O_2/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 Limit of detection (LOD) is calculated as: Xn+3SD and is determined under optimal conditions.

Matrix	Sample preparation	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

4. HANDLING AND STORAGE

- Store the kit at +2ºC to +8ºC in a dark place. For repeated use store kit components as specified under chapter 9.
- After the expiry date (see kit label) has passed, quality claims are not accepted.
- Before opening the sealed plate, the plate should be at ambient temperature in order to avoid condensation in the ELISA.
- Dilute the kit components immediately before use, but after the components are at ambient temperature.
- The substrate chromogen solution can be stored in a refrigerator (+2ºC to + 8ºC) until the expiry date stated on the label.
- Any direct action of light on the substrate chromogen solution should be avoided.

Degeneration of the reagents may have occurred when the following phenomena are observed:

- A blue colouring of the substrate solution before transferring it into the wells.
- A weak or absent colour reaction of the zero standard (Bmax, E450nm < 0.8).

13. ORDERING INFORMATION

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

14. LAST MUTATIONS

Liver, red pepper, feed and serum samples are added.

EuroProxima B.V.
Beijerinckweg 18
NL 6827 BN Arnhem
The Netherlands

TEL: + 31 26 3630364
FAX: + 31 26 3645111
Web-site:http://www.europroxima.com
E-mail: info@europroxima.com

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

1. J.E. Smith, C.W. Lewis and J.G. Anderson. Mycotoxins in human nutrition and health. EU Directorate-General XII, Science, Research and Development, 1990.

2. J.L. Richard, G.A. Bennett, P.F. Ross and P.E. Nelson. Analysis of naturally occurring mycotoxins in feedstuffs and food. J. Anim. Sci. 71, 2563-2574, 1993.

3. Council Directive 1999/29EC of 22 April 1999 on the undesirable substances and products in animal nutrition. Off. J. European Commun. L115 (1999) 32-46.

4. Commission Regulation (EC) No 466/2001 of 8 March 2001 setting maximum levels for certain contaminants in foodstuffs. Off. J. European Commun. L77 (2001) 1-13.

5. Commission Regulation (EC) No 472/2002 of 12 March 2002 amending Regulation (EC) No 466/2001 setting maximum levels for certain contaminants in foodstuffs.

6. 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.

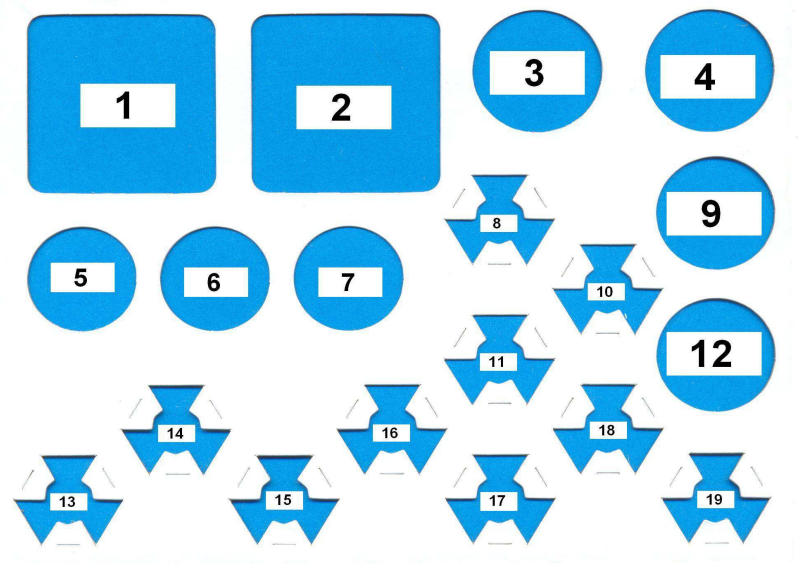
7. Commission Regulation (EC) No 683/2004 of 13 April 2004 amending Regulation (EC) No 466/2001 as regards aflatoxins and ochratoxin A in foods for infants and young children. Off. J. European Commun. L106 (2004) 3-5.

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 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
- 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

7. PRECAUTIONS

- Aflatoxins are toxic compounds. Avoid contact with mouth and skin. Be aware that aflatoxins are not inhaled.
- The stop reagent contains 0.5 M sulfuric acid. Do not allow the reagent to get into contact with the skin.
- 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.
- TMB is toxic by inhalation, in contact with skin and if swallowed; take care when handling the substrate.
- 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, which crystallises 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.

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.

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

Calibration curve:
The values (% 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.

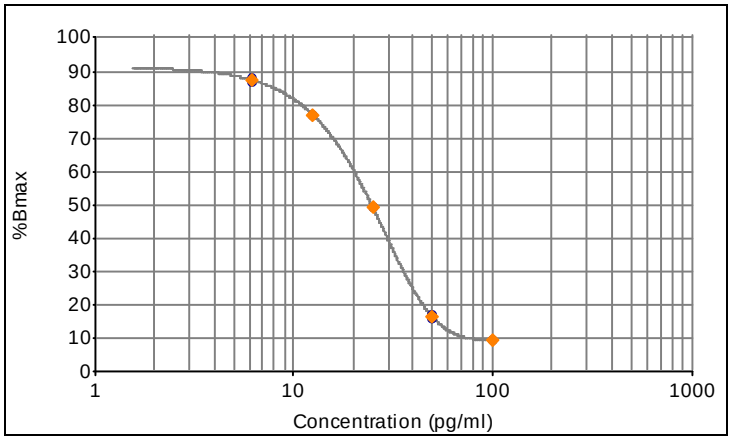


Figure 1 : Example of a calibration curve

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 % maximal absorbance of each extract can be read from the calibration curve.

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

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 (20ºC to 25ºC).

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 (20ºC to 25ºC).

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

14. Read the absorbance values immediately at 450 nm.

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
- 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
- Dilute with sample dilution buffer according to the factor per matrix as specified below
- Use an aliquot of 100 µl of the respective dilutions in the ELISA

- For **baby food, cereals or nuts**, pipette 100 µl of the layer underneath directly in the respective wells of the ELISA plate. Final dilution is 1 : 4.

- For **liver**, first dilute the layer underneath 1 : 2 in *sample dilution buffer. Then pipette 100 µl of this dilution in the respective wells of the ELISA plate. Final dilution is 1 : 8.

- For **red pepper**, first dilute the layer underneath 1 : 20 in *sample dilution buffer. Then pipette 100 µl of this dilution in the respective wells of the ELISA plate. Final dilution is 1 : 80.

- For **feed**. First dilute the layer underneath 1 : 100 in *sample dilution buffer. Then, pipette 100 µl of this dilution in the respective wells of the ELISA plate. Final dilution is 1 : 400.

- For **serum,** pipette 100 µl of the layer underneath directly in the respective wells of the ELISA plate. Final dilution is 1 : 4.

* See chapter 9

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 buffers
In this assay two dilution buffers are used. The dilution buffer for the conjugate is supplied ready-to-use in the kit (chapter 5 nr 1). The dilution buffer for samples and standards has to be prepared. For preparation see *Sample/Standard dilution buffer.

*Sample/standard dilution buffer (PBS/methanol)

Phosphate Buffered Saline (PBS)	1 litre
Na_2HPO_4	0.77 g
KH_2PO_4	0.18
NaCl	8.94
pH7.4 (7.2 – 7.5)	

Prepare fresh before use the final sample/standard dilution buffer. Add to 45 ml PBS, 5 ml of 100% methanol.

Standard solutions
Prepare a dilution range of the aflatoxin standard. 3 Vials of standard (lyophilised) 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 40 ml of diluted rinsing buffer is used (2 ml concentrated rinsing buffer + 38 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 (Sample preparation) and prepare reagents according to Chapter 9 (Preparation of reagents).

2. Pipette 100 µl of the *sample/standard dilution buffer in duplicate (wells G1, G2, blank).
   Pipette 100 µl of the *sample/standard dilution buffer (zero standard, Bmax) in duplicate (wells A1, A2).
   Pipette 100 µl 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 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.