FUMONISIN ELISA

5121FUM[12]07.20

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

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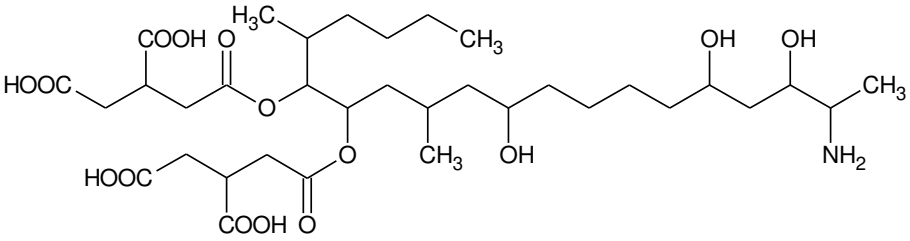
BRIEF INFORMATION

The fumonisin ELISA is a competitive enzyme immunoassay for measurement of the concentration of fumonisin. 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



Chemical structure of fumonisin B1

Fumonisins are mycotoxins produced primarily by *Fusarium moniliforme* and *F. proliferatum* in corn. The fumonisins B1, B2, A1, A2 are a structurally closely related group of mycotoxins resembling the structure of natural sphingolipids. Fumonisins have been implicated as the causal agents in a variety of animal diseases and are epidemiologically linked to the high incidence of human oesophageal cancer in some regions in the world. Fumonisins are considered to be responsible for two distinct diseases in animals, namely equine leucoencephalomalacia (ELEM) in horses and porcine pulmonary edema (PPE) in swine.
Contamination of corn with *F. moniliforme* has been associated with human oesophageal cancer in the Transkey region of South Africa.
Fumonisins are heat stable compounds that survive under most conditions used during baking and frying.
The maximum level in feed for animals is 5 mg/kg (5 ppm, horses), 10 mg/kg (10 ppm, swine) and 50 mg/kg (50 ppm, poultry and cattle).

2. PRINCIPLE OF THE FUMONISIN ELISA

The microtiter plate based fumonisin ELISA consists of one precoated plate (12 strips, 8 wells each). Antibody, Horseradish peroxidase (-HRP) labeled fumonisin and standard solution or sample are added to the wells. Free fumonisin from the samples or standards and fumonisin-HRP conjugate compete for the specific antibody binding sites (competitive enzyme immunoassay).
After an incubation step of one hour, the non-bound reagents are removed in a washing step. The amount of bound fumonisin-HRP conjugate is visualized by the addition of a substrate/chromogen solution (H_2O_2/TMB). Bound fumonisin-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 fumonisin concentration in the sample.

3. SPECIFICITY AND SENSITIVITY

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

Cross-reactivities:

Fumunisin B1	100%
Fumonisin B2	27%
Fumonisin B3	76%

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)
Corn	8.1	2
Milk	8.2	1
Honey	8.3	2
Serum	8.4	2

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:

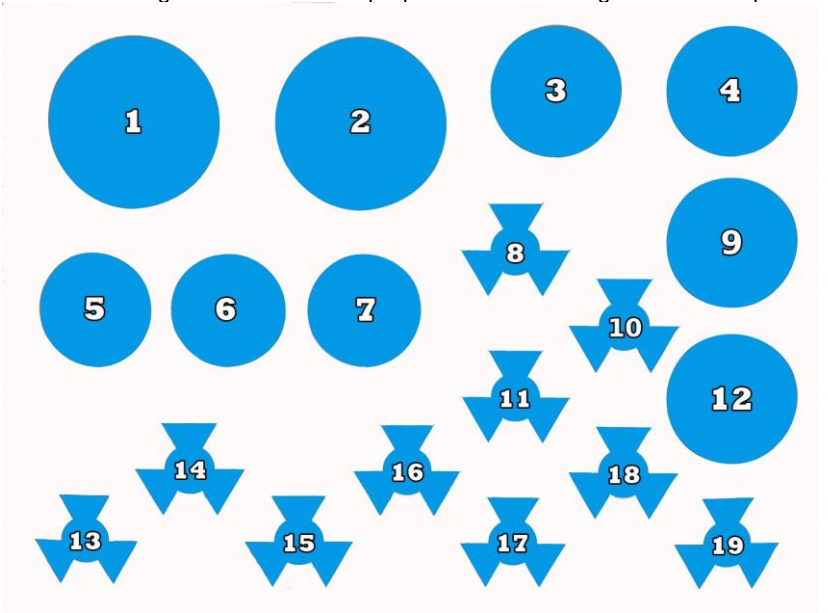
- A blue colouring of the chromogen solution before transferring it into the wells.
- A weak or no colour reaction in the zero standard wells (E450nm < 0.8).

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. **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. not in use
6. **Antibody solution** (4 ml, Ready-to-use)
7. not in use
8. **Conjugate Solution** (150 µl, 100x concentrated)
9. not in use
10. not in use
11. not in use
12. not in use
13. **Zero standard** (2ml, Ready-to-use)
14. **Standard solution 1 (**1ml, Ready-to-use) **0.125 ng/ml**
15. **Standard solution 2** (1ml, Ready-to-use) **0.25 ng/ml**
16. **Standard solution 3** (1ml, Ready-to-use) **0.5 ng/ml**
17. **Standard solution 4** (1ml, Ready-to-use) **1.0 ng/ml**
18. **Standard solution 5** (1ml, Ready-to-use) **2.0 ng/ml**
19. **Standard solution 6** (1ml, Ready-to-use) **4.0 ng/ml**

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
- Micropipettes, 100 – 1000 µl
- Multipipette with 2.5 ml combitips
- Methanol 100%
- 4 ml glass tubes
- 15 ml tubes with screw cap (polypropylene)

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 Corn samples
- Approximately 50 - 100 g of sample is ground and pulverised into a fine powder.
- An amount of 3 g of ground sample is extracted with 9 ml of 80% methanol.
- Mix at room temperature head over head for 15 minutes.
- Centrifuged (10 minutes, 2000 x g) or filtrated (recommended for larger sample volumes).
- An aliquot of 50 µl of the supernatant obtained after centrifugation, or an aliquot of 50 µl of filtrate obtained after filtration, is diluted with 150 µl of dilution buffer to obtain a solution containing 20% methanol.
- Use 50 µl of this solution in the ELISA test.

8.2 Milk samples
The pH of the sample is a potential pitfall. Sour milk disturbs the ELISA, neutralization of the pH is essential.

Defat Procedure:
Centrifuge cold milk samples for 15 minutes at 2000 x g and at 4°C.
Remove the upper fat layer using a spatula.

- Transfer 0.5 ml of the defatted milk in a test tube.
- Add 4.5 ml of 20% methanol in dilution buffer and mix.
- Use 50 µl of this solution in the ELISA test

8.3 Honey samples
- Homogenize a representative amount of honey sample, if necessary, warm the honey for a better homogenization.
- Weigh in 3 g of honey and transfer into a tube.
- Add 9 ml 80% methanol/water distilled water and mix head over head for 30 minutes.
- Centrifuge 10 minutes, 2000 x g,
- Dilute 50 supernatant with 150 ul dilution buffer, vortex
- Ust 50 µl of this solution in the ELISA test .

8.4 Serum samples
- Samples are diluted 1:10 in dilution buffer.
- Use 50 µl of this solution is the ELISA test..

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.

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

Substrate solution
The substrate solution (ready to use) precipitates 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.

Conjugate solution
The conjugate is delivered 100x concentrated.
Dilute the concentrated conjugate (fumonisin B1-HRP) with dilution buffer, mix thoroughly and keep in the dark until use. Add 10 µl of conjugate to 990 µl of dilution buffer. Per 2 x 8 wells 400 µl is required. Store the unused concentrated conjugate in the dark at +2°C to +8°C.

Antibody solution :
The antibody solution is ready for use. Mix thoroughly and keep in the dark until use. Store the vial immediately after use in the dark at +2°C to +8°C.

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 the zero standard in duplicate (wells H1, H2, blank).
   Pipette 50 µl of the zero standard (zero standard, Bmax) in duplicate (wells A1, A2).
   Pipette 50 µl of each standard solution in duplicate (wells B1,2 to G1,2 i.e. 0.125, 0.25, 0.5. 1.0, 2.0 and 4.0 ng/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 (fumonisin-HRP) to all wells, except H1 and H2.

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

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

7. Incubate for 1 hour in the dark at 37°C.

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 at room temperature (20ºC to 25°C) in the dark.

11. Pipette 100 µl of stop solution into 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.

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

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

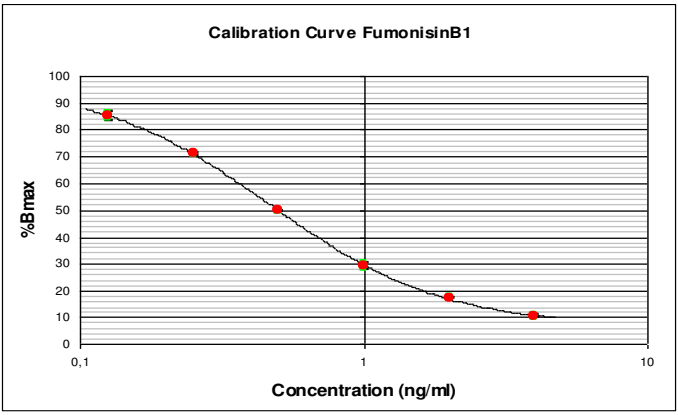


Figure 1: Example of a calibration curve

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

Corn samples
The fumonisin equivalents read from the calibration curve, have to be multiplied by a factor 16.

Milk samples
The fumonisin equivalents read from the calibration curve, have to be multiplied by a factor 10.

Honey samples
The fumonisin equivalents read from the calibration curve, have to be multiplied by a factor 16.

Serum samples
The fumonisin equivalents read from the calibration curve, have to be multiplied by a factor 10.

12. LITERATURE

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Jackson L.S., Katta S.K., Fingerhut D.D., De Vries J.W. and Bullerman L.B. Effects of baking and frying on the Fumonisin B1 content of corn-based foods. J. Agr. Food Chem. 45 (12), 4800-4805, 1997.

13. ORDERING INFORMATION

For ordering the Fumonisin B1 ELISA kit, please use cat. code 5121FUM.

14. REVISION HISTORY

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

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