Importance of Aflatoxin M_1 Determination

Aflatoxins are highly toxic mycotoxins produced by a variety of molds such as *Aspergillus flavus, A. parasiticus* and *A. nomius.* These toxins are known carcinogens and can be present in grains, nuts, cottonseed and other foods consumed by humans or in animal feed.

Crops may be contaminated by some of these toxins during growth, harvest or storage. The toxins most frequently detected are Aflatoxin B_1, B_2, G_1, and G_2. When animals are fed contaminated feed, Aflatoxin B_1 is converted to M_1 by hydroxylation, which is subsequently secreted into the milk of lactating animals. Human breast milk can also contain Aflatoxin M_1 if a lactating woman has consumed food contaminated with Aflatoxin B_1.

Aflatoxin M_1 is very stable throuhout milk processing methods such as pasteurazation.

To protect humans, regulatory agencies around the world have imposed regulatory limits regarding the amount of Aflatoxins that are allowable in human and animal foods. In Europe, the Aflatoxin M_1 tolerance levels (ML) are as follows:

Milk (raw milk, milk used in the production of milk based products and heat treated milk): 0.05 ppb
Infant formula and infant milk: 0.025 ppb
Dietary foods intended for infants: 0.025 ppb

The Aflatoxin M_1 ELISA allows the determination of 42 samples in duplicate determination. Less than 1 mL of sample extract is required. The test can be performed in less than 2 hours.

Performance Data

Test sensitivity: The detection limit for Aflatoxin M_1 is 11 pg/mL (mean of 6 blank determinations minus 3 standard deviations). The middle of the test (50% B/B_0) is at approximately 100 pg/mL. Determinations closer to the middle of the calibration curve give the most accurate results.

Test reproducibility: Coefficients of variation (CVs) for standards: <10%; CVs for samples: <15%.

Specificity: The cross-reactivity of the Abraxis Aflatoxin M_1 Kit for various other Aflatoxins (B_1, B_2, G_1, and G_2) in milk were not determined as these mycotoxins are not excreted in milk.

Recoveries: Recoveries of Aflatoxin M_1 from spiked whole milk are as follows:

M_1 Spiked (ppt)	M_1 Recovered (ppt)	Recovery (%)
50	50.3	100.6
100	104.6	104.6
200	188.1	94.1
Average Recovery		99.8

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ABRAXIS

Aflatoxin M_1 ELISA (Microtiter Plate)

Enzyme-Linked Immunosorbent Assay for the Determination of Aflatoxin M1 in Contaminated Samples

Product No. 53012M

1. General Description

The Aflatoxin M1 ELISA is an immunoassay for the quantitative and sensitive screening of Aflatoxin M_1. This test is suitable for the quantitative and/or qualitative screening of Aflatoxin M_1 in milk and milk products (please refer to the appropriate technical bulletins for extraction/dilution procedures). Samples requiring regulatory action should be confirmed by HPLC, GC/MS, or other conventional methods.

2. Safety Instructions

The standard solutions of the test kit contain small amounts of Aflatoxin M_1. In addition, the substrate solution contains tetramethylbenzidine and the stop solution contains diluted sulfuric acid. Avoid contact of stopping solution with skin and mucous membranes. If these reagents come in contact with the skin, wash with water.

3. Storage and Stability

The Aflatoxin M_1 ELISA should be stored in the refrigerator (4–8°C). The solutions must be allowed to reach room temperature (20-25°C) before use. Reagents may be used until the expiration date on the box.

4. Test Principle

The test is a forward ELISA and it is based on the recognition of Aflatoxin M_1 by antibodies. The calibrators and sample extract(s) are pipetted into test wells coated with Aflatoxin M_1 antibody to initiate the reaction. After a 30 minute incubation and washing of the wells, Aflatoxin-M_1 HRP conjugate is added, followed by a 60 minute incubation period. The HRP conjugate binds to unbound sites on the Aflatoxin M_1 antibody. Following this 60 minute incubation, the contents of the well are removed and the wells are washed to remove any unbound Aflatoxin M_1 HRP conjugate. After washing with the diluted wash solution, a clear substrate is added to the wells and any bound enzyme conjugate causes the conversion of the colorless substrate to a blue color. Following a 20 minute incubation, the reaction is stopped and the amount of color in each well is read using an ELISA reader. The color of the unknown samples is compared to the color of the calibrators and the Aflatoxin M_1 concentration of the samples is derived. The concentrations of the samples are determined by interpolation using the standard curve constructed with each run.

5. Limitations of the Aflatoxin M_1 ELISA, Possible Test Interference

Although many organic and inorganic compounds commonly found in samples have been tested and found not to interfere with this test, due to the high variability of compounds that might be found in samples, test interferences caused by matrix effects can't be completely excluded. Milk fats will cause interference in the test, therefore milk samples should be defatted as instructed in the sample preparation step (Section H) before testing in the ELISA.

Mistakes in handling the test also can cause errors. Possible sources for such errors can be: inadequate storage conditions of the test kit, wrong pipetting sequence or inaccurate volumes of the reagents, too long or too short incubation times during the immune and/or substrate reaction, extreme outside temperatures (lower than 10 ^{0}C or higher than 30 ^{0}C) during the test performance.

The Abraxis Aflatoxin M_1 ELISA kit provides screening results. As with any analytical technique (GC, HPLC, etc.), samples requiring some regulatory action should be confirmed by an alternative method.

A. Reagents and Materials Provided

1. Microtiter plate (12 X 8 strips) coated with polyclonal anti-Aflatoxin M_1 antibody, in an resealable aluminum pouch
2. Calibrators/Standards (6): 0, 15, 25, 50, 100, 250 pg/mL (ppt) of Aflatotoxin M_1, 1 mL each
3. Aflatoxin M_1-HRP Conjugate, 12 mL
4. Wash Solution (5X) Concentrate, 100 mL. Must be diluted 1:5 with deionized water before use
5. Substrate (Color) Solution (TMB), 12 mL
6. Stop Solution, 6 mL (Handle with care)

B. Test Preparation

Micro-pipetting equipment and pipette tips for pipetting the standards and the samples are necessary. We recommend using a multi-channel pipette or a stepping pipette for adding the enzyme conjugate, the substrate solution and the stop solution in order to equalize the incubation periods on the entire microtiter plate. Please only use the reagents and standards from one package lot in one test, as they have been adjusted in combination.

1. Adjust the microtiter plate and the reagents to room temperature before use.
2. Remove the number of microtiter plate strips required from the aluminum pouch. The remaining strips are stored in the aluminum pouch and zip-locked closed. Store the remaining kit in the refrigerator (4-8°C).
3. The standard solutions, enzyme conjugate, substrate and stop solution are ready to use and do not require any further dilutions.
4. Dilute the wash buffer at a ratio of 1:5. If using the entire bottle (100 mL), add to 400 mL of deionized or distilled water.
5. The stop solution must be handled with care as it contains diluted H_2SO_4.

C. Assay Procedure

1. Add 100 µL of the **standard solutions** or the **sample extracts (Section H)** into the wells of the test strips according to the working scheme given. We recommend using duplicates or triplicates.
2. Incubate for 30 minutes at room temperature.
3. Wash the strips three times using the diluted **washing buffer solution**. Please use at least a volume of 250 µL of washing buffer for each well and each washing step. Remaining buffer in the wells should be removed by patting the plate dry on a stack of paper towels.
4. Add 100 µL of **enzyme conjugate** solution to the individual wells successively using a multi- channel pipette or a stepping pipette.
5. Incubate the strips for 60 min at room temperature.
6. Wash the strips three times using the diluted **washing buffer solution**. Please use at least a volume of 250 µL of washing buffer for each well and each washing step. Remaining buffer in the wells should be removed by patting the plate dry on a stack of paper towels.
7. Add 100 µL of **substrate (color) solution** to the wells. Incubate the strips for 20 min at room temperature. Protect the strips from direct sunlight.
8. Add 50 µL of **stop solution** to the wells in the same sequence as for the substrate solution.
9. Read the absorbance at 450 nm using a microplate ELISA photometer within 15 minutes after the addition of stopping solution.

D. Evaluation

The evaluation of the ELISA can be performed using commercial ELISA evaluation programs such as Logit/Log or 4-Parameter (preferred). For a manual evaluation, calculate the mean absorbance value for each of the standards. Calculate the $\%B/B_0$ for each standard by dividing the mean absorbance value for each standard by the Zero Standard (Standard 0) mean absorbance. Construct a standard curve by plotting the $\%B/B_0$ for each standard on the vertical linear (y) axis versus the corresponding Aflatoxin M_1 concentration on the horizontal logarithmic (x) axis on graph paper. $\%B/B_0$ for samples will then yield levels in ppt or pg/mL of Aflatoxin M_1 by interpolation using the standard curve. Results can also be obtained by using a spreadsheet macro available from Abraxis upon request.

The concentrations of the samples are determined using the standard curve run with each test. Samples showing a lower concentrations of Aflatoxin M_1 compared to standard 1 (15 pg/mL or ppt) must be reported as containing < 15 ppt Aflatoxin M_1. Samples showing a higher concentration than standard 5 (250 pg/mL) must be diluted further to obtain accurate results.

Semi-quantitative results can be derived by simple comparison of the sample absorbances to the absorbance of the calibrators. Sample containing less color than a calibrator will have a concentration of Aflatoxin M_1 greater than the concentration of the calibrator. Samples containing more color than a calibrator will have a concentration less than the concentration of the calibrator.

E. Additional Materials (not delivered with the test kit)

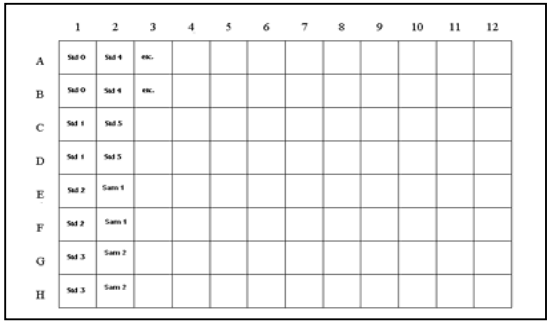
1. Micro-pipettes with disposable plastic tips (50-200 µL)
2. Multi-channel pipette (50-250 µL) or stepper pipette with plastic tips (50-250 µL)
3. Microtiter plate washer (optional)
4. Microtiter plate reader (wave length 450 nm)
5. Shaker for microtiter plates (optional)
6. Deionized or distilled water
7. Paper towels or equivalent absorbent material
8. Timer
9. Microcentrifuge capable of spinning at 12,000 – 14,000 RPM
10. Microcentrifuge tubes

F. Working Scheme

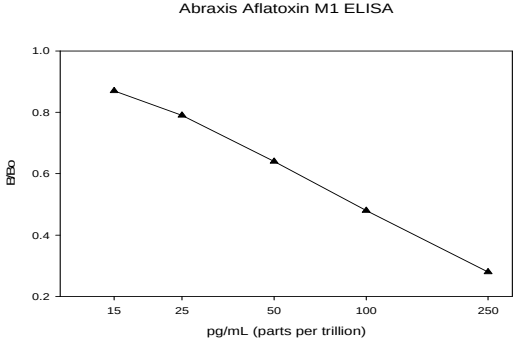
The microtiter plate consists of 12 strips of 8 wells, which can be used individually for the test. The standards must be run with each test. Never use the values of standards which have been determined in a test performed previously.

Std 0-Std 5: Standards
(0; 15; 25; 50; 100; 250 ppt)

Sam1, Sam2, etc.: Samples



G. Standard Curve (These values are used for demonstration purposes; do not use these values for your determinations)



H. Milk Sample Extraction

1. Pipette 1 mL of milk into a centrifuge tube, centrifuge at 12,000 – 14,000 RPM for 5 minutes.
2. Using a pipette, carefully remove the **middle** layer (the milk fat is the top layer).
3. Run 100 uL of the **middle** layer directly in the assay (step 1 on Assay Procedure).
4. Highly contaminated samples (samples outside the standard curve range) should be diluted in order to get the value in the middle of the curve and re-analyzed.

I. Powder Milk Extraction (non-fat)

1. Weigh 1 gm of milk powder, add 10 mL of DI water, shake to dissolve. Analyze directly in the ELISA (step 1 Assay Procedure). Assay results will need to be multiplied by 10.

J. Powder Milk (regular)

1. Weigh 1 gm of milk powder and dilute with 10 mL of DI water, shake to dissolve.
2. Centrifuge at 12,000 – 14,000 RPM for 5 minutes.
3. Follow same steps as H. 3-4. Assay results will need to be multiplied by 10.