



## Test Instruction

Aflatoxin M<sub>1</sub>

96 Tests

Enzyme Immunoassay  
for the Quantitative  
Determination of Aflatoxin M<sub>1</sub>  
in Milk and Milk Products

Cat.-No.: AM1-E01

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**Immunolab GmbH**

Otto-Hahn-Str. 16  
D-34123 Kassel  
Germany

Phone: +49 (0) 561 491742-0  
Fax: +49 (0) 561 491742-20  
E-Mail: [info@immunolab.de](mailto:info@immunolab.de)

[www.immunolab.de](http://www.immunolab.de)

|                   |           |
|-------------------|-----------|
| Sensitivity       | <10 pg/mL |
| Recovery (Milk)   | 102%      |
| Recovery (Cheese) | 60%       |
| Incubation Time   | 140 min   |

### General Information

Aflatoxins belong to the class of mycotoxins. Chemically they are defined as difuranocyclopentanocumarines or difuranopentanolidocumarines, i.e. aflatoxins contain a dihydrofuran or a tetrahydrofuran ring, to which a substituted cumarin system is condensed. Out of about 20 known aflatoxins, the moulds *Aspergillus flavus* and *A. parasiticus* produce exclusively aflatoxin B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub> and G<sub>2</sub>, and all the other aflatoxins are derivatives of these four. The derivatives are developed either by metabolism in humans, animals and microorganisms or by environmental reactions.

Aflatoxin M<sub>1</sub> was the first metabolite of Aflatoxin B<sub>1</sub>, which could unequivocally be detected by Allcroft and Carnaghan in the milk of cows in 1963. Out of this reason this first derivative was called Aflatoxin M<sub>1</sub> (= milk). As further investigations showed, also other mammals excrete Aflatoxin M<sub>1</sub> in milk, feces and urine. Contaminations of milk and milk products can be hazardous for human beings, because M<sub>1</sub> is similar to Aflatoxin B<sub>1</sub> regarding its hepatotoxicity. M<sub>1</sub> is only less carcinogenic.

In order to protect people against aflatoxin-induced diseases, there is a need for the qualitative and quantitative control of endangered foodstuff, besides appropriate hygienic precautions, which avoid the formation of aflatoxins. The **Immunolab Aflatoxin M<sub>1</sub> ELISA** is a quick, economical and sensitive method to detect aflatoxin M<sub>1</sub> in milk and milk products. After an appropriate sample preparation, 40 samples can be tested in duplicate within 140 minutes.

### Principle of the Test

The **Immunolab Aflatoxin M<sub>1</sub>** quantitative test is based on the principle of the enzyme linked immunosorbent assay. An aflatoxin conjugate is bound on the surface of a microtiter plate. Aflatoxin M<sub>1</sub> containing samples or standards and an antibody directed against aflatoxin M<sub>1</sub> are given into the wells of the microtiter plate. Immobilized and free aflatoxin M<sub>1</sub> compete for the antibody binding sites. After one hour incubation at room temperature, the wells are washed with diluted washing solution to remove unbound material. A peroxidase conjugate against the antibody is given into the wells and after another hour incubation, the plate is washed again. Then a substrate solution is added and incubated for 20 minutes, resulting in the development of a blue colour. The colour development is inhibited by the addition of a stop solution, and the colour turns yellow. The yellow colour is measured photometrically at 450 nm. The concentration of aflatoxin M<sub>1</sub> is indi-

rectly proportional to the colour intensity of the test sample.

### Precautions

Full compliance of the following good laboratory practices (GLP) will determine the reliability of the results:

1. Prior to beginning the assay procedure, bring all reagents to room temperature (20-25°C).
2. All reagents should be mixed by gentle inversion or swirling prior to use. Do not induce foaming.
3. Once the assay has been started, all subsequent steps should be completed without interruption and within the recommended time limits.
4. Replace caps in all the reagents immediately after use. Do not interchange vial stoppers.
5. Use a separate disposable tip for each specimen to prevent cross-contamination.
6. All specimens and standards should be run at the same time, so that all conditions of testing are the same.
7. Do not mix components from different batches.
8. Do not use reagents after expiration date.
9. Check both precision and accuracy of the laboratory equipment used during the procedure (micropipets, ELISA reader etc.).

### Health and safety instructions

1. Do not smoke or eat or drink or pipet by mouth in the laboratory.
2. Wear disposable gloves whenever handling patient specimens.
3. Avoid contact of substrate and stop solution with skin and mucosa (possible irritation, burn or toxicity hazard). In case of contact, rinse the affected zone with plenty of water.
4. Handling and disposal of chemical products must be done according to good laboratory practices (GLP).
5. Aflatoxins are very toxic substances. They can cause cancer or irreversible damages of the genetic substance. Aflatoxins are toxic after inhalation, swallowing or dermal contact. Appropriate protective clothing must be worn.

### Reagents

The kit contains reagents for 96 determinations. They have to be stored at 2-8°C. Expiry data are found on the labels of the bottles and the outer package.

1. Microtiter plate consisting of 12 strips with 8 breakable wells each, coated with aflatoxin conjugate.
2. Aflatoxin M<sub>1</sub> Standards (0; 100; 500; 1000; 5000; 10000 pg/mL): 6 vials with 0.5 mL each in methanol as 10x concentrate. Dilute 1+9 with sample/standard diluent.  
**Note: The concentrations above refer to the 10x concentrated standards.**
3. Anti-Aflatoxin M<sub>1</sub> Antibody (rabbit): 6 mL, dyed red, ready-to-use.
4. Conjugate (anti-rabbit-IgG-HRP): 15 mL, dyed red, ready-to-use.
5. Substrate Solution (TMB): 15 mL; ready-to-use.
6. Stop Solution (0.5 M H<sub>2</sub>SO<sub>4</sub>): 15 mL; ready-to-use.
7. Sample/Standard Diluent (PBS): 60 mL, dyed red, ready-to-use.
8. Washing Solution (PBS + Tween 20): 60 mL as 10x concentrate, dyed blue. Dilute 1+9 with distilled water. If during the cold storage crystals precipitate, the concentrate should be warmed up to 37°C for 15 minutes.
9. Two plastic foils to cover the strips during the incubation.
10. Plastic bag to store unused microtiter strips.
11. Instruction Manual.

### Additional Instrumentation and Reagents (not provided)

#### Instrumentation

- 50, 100, 500 and 1000 µL-micropipets
- Microtiter plate shaker
- ELISA reader (450 nm)
- Volumetric flask
- Mixer
- Horizontal shaker, magnetic stirrer
- Centrifuge

## Reagents

- Methanol
- Hexane
- Double-distilled water

## Sample Preparation

### Milk

- 5 mL of a fresh milk sample (full-cream milk or skim milk) are pipetted into a test tube and incubated for 30 minutes at 4°C.
- Afterwards there follows a centrifugation at 3000 g for 10 minutes. 450 µL of the clear milk serum below the fat layer is taken off and mixed with 50 µL methanol.
- This solution can now be directly inserted in the ELISA.

### Milk Powder

- 9.1 g skim milk powder or 12.5 g full-cream milk powder respectively are reconstituted with double-distilled water, so that the total volume comes to 100 mL, are further warmed up to about 50°C and are homogenized by using a magnetic stirrer and are finally treated according to the sample preparation for milk.

### Cheese

- A representative cheese sample is crushed in a mixer without addition of liquid.
- From this sample, 2 g cheese are combined with 10 mL of a mixture of hexane, methanol and double-distilled water (50:30:20) and are extracted for 30 minutes on a horizontal shaker at 125 / minute.
- The liquid is decanted and centrifuged for 5 minutes at 3000 g.
- The lower aqueous methanolic phase is removed by means of a pasteur pipette, diluted 1:10 with sample diluent and then directly inserted in the ELISA.

Alternatively also immunoaffinity columns (Cat.-No.: AFM-A01) can be employed for the extraction. When using such columns care must be taken, that the eluate which is given into the **Immunolab Aflatoxin M<sub>1</sub> ELISA** does not contain more than 5% organic solvent (methanol, acetone). In that case, the eluate must be further diluted with sample diluent.

## Reagent Preparation

Because the standards are concentrated 10x, they have to be diluted by the enclosed standard/sample diluent 1:10 (e.g. 50 µL standard + 450 µL diluent), before using them in the assay procedure.

## Procedure

1. Prepare samples as described above.

2. Pipet 100 µL diluted (1:10) standards or prepared samples in duplicate into the appropriate wells of the microtiter plate. Immediately add 50 µL aflatoxin M<sub>1</sub> antibody into each well.
3. Cover the microtiter plate with a plastic foil and incubate for 60 minutes at room temperature on a microtiter plate shaker (or 90 minutes without shaker).
4. Wash the plate three times as follows: Pipet 300 µL of diluted washing solution into each well. Discard the contents of the wells by turning the plate over and rapidly striking the plate against a paper towel. Repeat the above procedure three times. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbances.
5. Pipet 100 µL of conjugate (anti-rabbit-IgG-HRP) into each well.
6. Cover the microtiter plate with a plastic foil and incubate for 60 minutes at room temperature on a microtiter plate shaker (or 90 minutes without shaker).
7. Wash the plate as outlined in 4.
8. Pipet 100 µL of substrate solution into each well.
9. Allow the reaction to develop in the dark (e.g. cupboard or drawer; the chromogen is light-sensitive) for 20 minutes at room temperature.
10. Stop enzyme reaction by adding 100 µL of stop solution (0.5 M H<sub>2</sub>SO<sub>4</sub>) into each well. The blue colour will turn yellow upon addition.
11. After thorough mixing, measure absorbance at 450 nm (reference wavelength 620 nm), using an ELISA reader. The colour is stable for 30 minutes.

## Calculation of results

1. Calculate the average optical density (OD 450 nm) for each set of reference standards or samples.
2. Construct a standard curve by plotting the mean optical density obtained for each reference standard against its concentration in pg/mL on semi-log graph paper with the optical density on the vertical (y) axis and the concentration on the horizontal (x) axis.
3. Using the mean optical density value for each sample, determine the corresponding concentra-

tion of aflatoxin M<sub>1</sub> in pg/mL from the standard curve. Depending on experience and/or the availability of computer capability, other methods of data reduction may be employed.

- The diluted samples must be further converted by the appropriate dilution factor. The dilution factor for milk(powder) is 1 and for cheese 25 according to the sample preparation procedure as described above.

### Typical Standard Values

The following table contains an example for a typical standard curve. The binding is calculated as percent of the absorption of the 0 pg/mL standard. These values are only an example and should not be used instead of the standard curve which has to be measured in every new test.

| Aflotoxin M <sub>1</sub> (pg/mL) | (% binding of 0 ng/mL) |
|----------------------------------|------------------------|
| 0                                | 100                    |
| 10                               | 90                     |
| 50                               | 84                     |
| 100                              | 78                     |
| 500                              | 47                     |
| 1000                             | 34                     |

### Performance

#### Sensitivity

The sensitivity of the **Immunolab Aflatoxin M<sub>1</sub> ELISA** is <10 pg/mL (based on the standard curve).

#### Recovery

The recovery of spiked samples was determined to 102% for milk and 60% for cheese.

#### Intra-assay Precision

The intra-assay variation of the aflatoxin M<sub>1</sub> test was determined to 3%.

#### Cross-reactivity relative to aflatoxin M<sub>1</sub> (=100%)

|                          |      |
|--------------------------|------|
| Aflatoxin B <sub>1</sub> | 10%  |
| Aflatoxin G <sub>1</sub> | 5%   |
| Aflatoxin B <sub>2</sub> | 3.5% |
| Aflatoxin G <sub>2</sub> | 2.1% |
| Sterigmatocystin         | 0.3% |

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