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biosystems inc

AFLATOXIN M₁ CAT. NO. 961AFLM01M-96

Competitive ELISA Immunoassay for the quantitative detection of Aflatoxin M₁ in milk, milk powder and cheese.

General

Aflatoxins are toxic metabolites produced by a variety of molds such as *Aspergillus flavus* and *Aspergillus parasiticus*. They are carcinogenic and can be present in grains, nuts, cottonseed and other commodities associated with human food or animal feeds. Crops may be contaminated by one or more of the four following sub-types of aflatoxin: B₁, B₂, G₁ and G₂. Aflatoxin B₁ is the most toxic and frequently detected form. The other types present a significant danger if the concentration is at a high level. Aflatoxins have been implicated in human health disorders including hepatocellular carcinoma, aflatoxicosis, Reye's syndrome and chronic hepatitis. Animals are exposed to aflatoxins by consumption of feeds that are contaminated by aflatoxin-producing fungal strains during growth, harvest or

storage. When cows are fed contaminated feed, aflatoxin B₁ is converted by hydroxylation to aflatoxin M₁, which is subsequently secreted in the milk of lactating cows. Aflatoxin M₁ is quite stable towards the normal milk processing methods such as pasteurization and if present in raw milk, it may persist into final products for human consumption. Most controlling government agencies worldwide have regulations regarding the amount of aflatoxins allowable in human and animal foodstuffs. Many countries have declared limits for the presence of aflatoxin M₁ in milk and milk products. In the EU the limit for the presence of M₁ in milk and reconstituted milk powders has been set at 0.05mg/L or 50 parts per trillion (50ppt.)

1. Intended Use

The HELICA Aflatoxin M₁ Assay is intended for the quantitative detection of Aflatoxin M₁ in milk, reconstituted milk powders and cheese.

2. Assay Principle

The HELICA Aflatoxin M₁ Assay is a solid phase competitive enzyme immunoassay. An antibody with a high affinity for aflatoxin M₁ is coated onto polystyrene microwells. Standard or sample is added to the appropriate well and if Aflatoxin M₁ is present it will bind to the coated antibody. Subsequently, aflatoxin bound to horse – radish peroxidase (HRP) is added and binds to the antibody not already occupied by aflatoxin M₁ present in the sample or standard. After this incubation period, the contents of the wells are decanted, washed and an HRP substrate is added which develops a blue color in the presence of enzyme. The intensity of the color is directly

proportional to the amount of bound conjugate and inversely proportional to the amount of aflatoxin M₁ in the standard or sample. Therefore, as the concentration of aflatoxin M₁ in the sample or standard increases, the intensity of the blue color will decrease. An acidic stop solution is added which changes the chromogen color from blue to yellow. The microwells are measured optically by a microplate reader with an absorbance filter of 450nm (OD₄₅₀). The optical densities of the samples are compared to the OD's of the kit standards and an interpolated result is determined.

3. Reagents Provided

1×pouch	Antibody coated microwell plate		96 wells (12×8) well holder coated with a mouse anti-aflatoxin monoclonal antibody
6×vials	Aflatoxin M ₁ Standards		3.0mL/vial of Aflatoxin M ₁ at the following concentrations: 0.0,5.0,10.0, 25.0, 50.0,100.0pg/mL(ppt) in stabilized skim milk
1×bottle	Aflatoxin HRP-conjugate	Green cap	12mL of aflatoxin conjugated to horseradish peroxidase in buffer with preservative, <i>Ready-to-Use</i>
1×bottle	Substrate Reagent	Blue cap	12mL stabilized urea peroxide and tetramethylbenzidine(TMB) – <i>Ready-to-Use</i>
1×bottle	Stop Solution	Red cap	12mL Acidic Solution, <i>Ready-to-Use</i>
1×pouch	Washing Buffer		PBS with 0.05% Tween20®, bring to 1 liter with distilled water and store refrigerated
1×bottle	M ₁ free skim milk	White cap	12mL skim milk for preparation of cheese extract

4. Materials Required but not Provided

- Single or multi-channel pipettor with 100 and 200µL tips
- Glass tubes
- Timer
- Wash bottle
- Absorbent paper towels
- Centrifuge
- Microplate reader with 450 nm filter

5. Warnings and Precautions for Users

1. Bring all reagents to room temperature (19°C - 25°C) before use.
2. Store reagents at 2°C to 8°C, and do not use beyond expiration date(s). Never freeze kit components.
3. Do not return unused reagents back into their original bottles. The assay procedure details volumes required.
4. Adhere to all time and temperature conditions stated in the procedure.
5. Never pipette reagents or samples by mouth.
6. The Stop Solution contains acid. Do not allow to contact skin or eyes. If exposed, flush with water.
7. Consider all materials, containers and devices that are exposed to sample or standards to be contaminated with aflatoxin M₁. Wear protective gloves when using this kit.
8. Dispose of all materials, containers and devices in an appropriate receptacle after use.
9. HRP-labeled conjugate and TMB-substrate are photosensitive and are packaged in a protective opaque bottle. Store in the dark and return to storage after use.

6. Preparation of Samples

Raw Milk

1. The standards are presented in homogenized skim milk and skim milk (milk plasma) is the appropriate sample for the assay.
2. An aliquot of unprocessed raw fatty milk should be placed at refrigerated temperature overnight to allow the fat globules to rise to the surface in a natural "creaming" effect. Centrifugation at this point is not necessary.
3. Alternatively, if the sample is at ambient temperature or has been mixed in transit, place an aliquot at refrigerated temperature for 1 – 2 hours and centrifuge at 2,000g for 5 minutes to induce separation of upper fatty layer.
4. Remove the upper fatty layer by aspiration and use the lower plasma in the assay.

Homogenized Milk

1. Homogenized skim milk should be used directly in the assay.
2. Due to the stabilization of the fat globules induced by the homogenizing process they are difficult to eliminate even by high speed centrifugation to create a plasma from homogenized fatty milk. Therefore used homogenized fatty milk directly in the assay. (see recovery data below)

Milk Powder

1. Reconstitute milk powders according to the manufacturer's instructions and treat the reconstituted product as above.

Cheese

1. One gram of finely grated or otherwise macerated cheese is mixed with 5mL of absolute methanol in a capped tube and mixed for 5 minutes. The tube is clarified by centrifugation (5,000g for 5 minutes) and the supernatant removed.
2. 0.5mL of this supernatant is transferred to a glass tube and the methanol evaporated by a stream of air (better recovery with nitrogen gas). This procedure results in the deposition of a semi-solid viscous material on the inside of the tube. Add 0.5mL of the provided blank skim milk to the tube and vortex vigorously for 1 minute. Allow the tube to stand for a further 5 minutes and use 2×200µL of this milk extract in the assay.

7. Assay Procedure

1. Bring the reagents to room temperature before use.
2. Unseal the pouch and remove the required number of wells for the number of standards and samples to be tested.
3. Return unused wells to the pouch and re-seal with tape to avoid the entry of moisture. (After the assay retain the well holder for future use.)
4. Using a fresh pipette tip for each, dispense 200µL aliquots of standards and samples into the appropriate wells in duplicate.
5. Cover the plate with sealing tape to avoid evaporation and protect from excess UV light.
6. Incubate at ambient temperature (19°C - 25°C) for 2 hrs.
7. Discard the contents of the wells into an appropriate receptacle. Wash the wells by filling with PBS-Tween 20 ® from a wash bottle or multi-channel pipette and immediately discard the washings into an appropriate receptacle. Repeat for a total of three washings. Tap the wells face down on a layer of absorbent paper to remove residual wash buffer.

8. Add 100µL of the conjugate (green cap) to each well.
9. Re-seal the plate and incubate at ambient temperature for 15 minutes.
10. Repeat step 7.
11. Add 100µL of enzyme substrate (TMB) to each well and incubate for 15 minutes. Cover to avoid direct light.
12. Stop the reaction by adding 100µL stop solution. The blue color will change to yellow.
13. Read the optical density (OD) of each microwell with a micro plate reader at 450nm using an air blank or a differential filter of 630nm.

8. Alternative Incubation Procedure

The first incubation period, with standards and sample, may be performed overnight at refrigerated temperature (4°C -6°C) with noticeable improvement in inhibition of zero binding for samples of 5ppt (ng/L) and above. If this option is chosen, the subsequent incubations with conjugate and TMB should be performed at ambient temperature.

INCUBATION TEMPERATURE		
ng/L	Overnight (4°C - 6°C) % binding	2 hours ambient % binding
0	100.0	100.0
5	83.8	89.0
10	67.5	80.1
25	37.4	55.5
50	19.8	36.3
100	12.5	21.2

Limit of detection (LOD) is defined as 2 standard deviations below the mean OD of multiple determinations of zero binding (n = 18, CV <2%) is 2ppt (ng/L) in both cases.

10. Interpretation of Results

Construct a dose-response curve using either the unmodified OD values or the OD values expressed as a percentage of the OD of the zero standard against the aflatoxin M₁ content of the standard. Unknowns are measured by interpolation from the standard curve.

The mean value of the absorbance values obtained for the standards and the samples are divided by the absorbance value of the zero standard and multiplied by 100. The zero standard is thus made equal to 100% and the absorbance values of other standards and samples are quoted in percentages of this value.

$$\text{absorbance standard (or sample) / absorbance zero standard} \times 100 = \text{\% absorbance}$$

The values calculated for the standards are entered in a system of coordinates on semilogarithmic graph paper against the aflatoxin M₁ concentration in ng/L. The aflatoxin M₁ concentration in ng/L corresponding to the absorbance of each sample can be read from the calibration curve.

In order to obtain the aflatoxin M₁ concentration in ng/L actually contained in a sample, the concentration read from the calibration curve must be further multiplied by the corresponding dilution factor. This is 1 for milk samples and 5 for cheese samples.

11. Specificity

The cross-reaction profile of the monoclonal antibody is as follows. M₁ 100%, B₁ 79%, B₂ 76%, G₁ 55%, M₂ 33%, G₂ 6%. While it is apparent that this antibody is not specific for aflatoxin M₁, the exclusive presence of aflatoxin M₁ in the standards together with the fact that M₁ is the predominant aflatoxin contaminant in cows milk means that it is functionally specific for the measurement of aflatoxin M₁ in this matrix. It should be noted that the cross-reactivity would detect any attempt at artificial contamination of the milk supply with the other more toxic B₁, B₂, G₁, and G₂ aflatoxins. A specific antibody would not detect this potentially catastrophic occurrence.

12. Recovery and Reproducibility

Recovery of 50ppt spiked into milk is as follows:

	% Recovery	Within Assay CV
SKIM MILK		
50ppt	100	4.2 %
1% FAT HOMOGENIZED		
50ppt	93	4.4 %
FULL FAT HOMOGENIZED		
50ppt	92	2.2%

Recovery in cheese: 100pg of aflatoxin M₁ was spiked into 1gm of finely grated parmesan cheese and allowed to remain in contact for one hour at ambient temperature. Applying the extraction procedure described above, the recovery of aflatoxin M₁ was 60.5% with a CV of 5.5% for 8 separate spiking/extractions.

Appendix

Performance Data

Sensitivity

<u>n</u>	<u>mean</u> <u>OD</u>	<u>SD</u>	<u>%CV</u>	<u>sensitivity</u>
18	1.857	0.023	1.2	2 ppt

Precision

Intra-assay

<u>sample</u> <u>(ppt)</u>	<u>n</u>	<u>mean</u> <u>OD</u>	<u>%B/B₀</u>	<u>% CV</u>
0	8	1.245	100	1.4
5	8	1.092	87.7	2.0
10	8	0.945	75.9	3.0
25	8	0.719	57.8	2.1
50	8	0.449	36.1	2.7
100	8	0.246	19.8	2.0

Inter-assay

over a seven month period with multiple lots

<u>sample</u> <u>(ppt)</u>	<u>n</u>	<u>%B/B₀</u>	<u>% CV</u>
5	8	86.6	1.5
10	8	76.2	3.3
25	8	55.5	4.7
50	8	36.3	4.8
100	8	22.0	10.2