Aflatoxin M1

ELISA Quantitative

Catalog # 961AFLM01M-96

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For the quantitative detection of Aflatoxin M_1 in milk, milk powder and cheese.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

Contents

Introduction

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: B_1, B_2, G_1 and G_2. Aflatoxin B_1 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 B_1 is converted by hydroxylation to Aflatoxin M_1, which is subsequently secreted in the milk of lactating cows. Aflatoxin M_1 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 M_1 in milk and milk products. In the EU the limit for the presence of M_1 in milk and reconstituted milk powders has been set at 50pg/mL or 50 parts per trillion (50ppt).

Assay Principle

The HELICA Aflatoxin M_1 Assay is a solid phase competitive enzyme immunoassay. An antibody with a high affinity for Aflatoxin M_1 is coated onto polystyrene microwells. Standard or sample is added to the appropriate well and if Aflatoxin M_1 is present it will bind to the coated antibody. Subsequently, aflatoxin bound to horseradish peroxidase (HRP) is added and binds to the antibody not already occupied by Aflatoxin M_1 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 an enzyme. The intensity of the color is directly proportional to the amount of bound conjugate and inversely proportional to the amount of Aflatoxin M_1 in the standard or sample. Therefore, as the concentration of Aflatoxin M_1 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 ODs of the kit standards and an interpolated result is determined.

Limitations Of The Procedure

- For research use only. Not for use in diagnostic procedures.
- Bring all reagents to room temperature (19-25°C) before use.
- Store reagents at 2-8°C, and do not use beyond expiration date(s). Never freeze kit components.
- Do not return unused reagents back into their original bottles. The assay procedure details volumes required.
- Adhere to all time and temperature conditions stated in the procedure.

Reagents Provided

1X pouch	**Antibody Coated Microwell Plate**	Silver Pouch	96 wells (12 eight-well strips) in a microwell holder coated with a mouse anti-Aflatoxin monoclonal antibody, *Ready-to-Use*
6 X vials	**Aflatoxin M_1 Standards**	White Cap (small)	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, *Ready -To-Use*
1X bottle	**Aflatoxin HRP-Conjugate**	Green Cap	12mL of Aflatoxin conjugated to peroxidase in buffer with preservative, *Ready -To-Use*
1X bottle	**Substrate Reagent**	Blue Cap	12mL stabilized tetramethylbenzidine (TMB), *Ready-To-Use*
1X bottle	**Stop Solution**	Red Cap	12mL acidic solution, *Ready-to-Use*
1X pouch	**Washing Buffer**	White Pouch	PBS with 0.05% Tween20® , bring to 1L with distilled water and store refrigerated
1X bottle	**M_1 Free Skim Milk**	White Cap (large)	12mL skim milk for preparation of cheese extract, *Ready-to-Use*

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 450nm filter

Precautions For User

- Never pipette reagents or samples by mouth.
- The Stop Solution contains acid. Do not allow to contact skin or eyes. If exposed, flush with water.
- Consider all materials, containers and devices that are exposed to sample or standards to be contaminated with Aflatoxin M_1. Wear protective gloves when using this kit.
- Dispose of all materials, containers and devices in an appropriate receptacle after use.
- 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.

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 the 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 use homogenized fatty milk directly in the assay. (see recovery data on page 7)

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 X 200µL of this milk extract in the assay.

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 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 (19-25ºC) 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 microplate reader at 450nm using an air blank or a differential filter of 630nm.

Alternative Incubation Procedure

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

Incubation Temperature		
Standard (pg/mL)	**%B/Bo Overnight** (4-6ºC)	**%B/Bo 2 Hours** Ambient
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 (pg/mL) in both cases.

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 M_1 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.

absorbance standard (or sample) / absorbance zero standard x 100 = % absorbance

The values calculated for the standards are entered in a system of coordinates on semilogarithmic graph paper against the Aflatoxin M_1 concentration

in pg/mL. The Aflatoxin M_1 concentration in pg/mL corresponding to the absorbance of each sample can be read from the calibration curve.

In order to obtain the Aflatoxin M_1 concentration in pg/mL 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.

Recovery And Reproducibility

Recovery of 50ppt spiked into milk is as follows:

Type of Milk	% 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 M_1 was spiked into 1g 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 M_1 was 60.5% with a CV of 5.5% for 8 separate spiking/extractions.

Performance Data

Sensitivity

n	Mean OD	SD	%CV	Sensitivity
18	1.857	0.023	1.2	2ppt

Precision

Intra-assay				
Sample (ppt)	**n**	**Mean OD**	**%B/Bo**	**% CV**
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			
Sample (ppt)	**n**	**%B/Bo**	**% CV**
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	10.8

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