AFLATOXIN M_1 2000

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Competitive ELISA Immunoassay for the quantitative detection of Aflatoxin M_1 in milk and milk powder.

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: 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 0.05 µg/L or 50 parts per trillion (50 ppt.) The equivalent value in the USA is 0.5 µg/L or 500 ppt.

1. Intended Use

The HELICA Aflatoxin M_1 Assay is intended for the quantitative detection of Aflatoxin M1 in milk, reconstituted milk powders and cheese.

2. 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 horse – radish 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 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 (OD_{450}). The optical densities of the samples are compared to the OD's of the kit standards and an interpretative result is determined.

3. Reagents Provided

1X pouch	Antibody coated microwells plate		96 wells (12X8-well strips) in a microwell holder, coated with a mouse anti-aflatoxin monoclonal antibody.
1 X Plate	Mixing Wells		96 wells (12X8-well strips), non-coated, in microwell holder. The wells are color coded green.
6X vials	Aflatoxin M_1 Standards		1.0mL/vial of Aflatoxin M_1 at the following concentrations: 0.0, 100.0, 250.0, 500.0, 1000.0, 2,000.0 pg/mL (ppt) in stabilized skim milk.
2X bottles	Assay Diluent	Brown cap	2×12mL
1X bottle	Aflatoxin HRP-conjugate	Green cap	12mL of aflatoxin conjugated to horseradish peroxidase in buffer with preservative, *Ready-to-Use*
1X bottle	Substrate Reagent	Blue cap	12mL stabilized urea peroxide and tetramethylbenzidine(TMB) – *Ready-to-Use*
1X bottle	Stop Solution	Red cap	12mL Acidic Solution, *Ready-to-Use*
1X 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 50, 100 and 200 µL 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 M_1. 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, use 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.

7. Assay Procedure

1. Bring the reagents to room temperature before use.
2. Place one mixing well in a microwell holder for each standard and sample to be tested. Place an equal number of antibody coated wells in a separate holder.
3. Return the unused wells to the pouch and re-seal to avoid the entry of moisture. Retain the well holder for future use.
4. Dispense 200µL of the assay diluent into each mixing well.
5. Using a fresh pipette tip for each, dispense 50µL of standards and samples into the appropriate wells and mix by aspirating three times.
6. Using a multichannel pipette, transfer 100µL of the mixture to the appropriate assay well and incubate at ambient temperature for 10 minutes. Note that the mixing wells contain enough for 100µL to be run in duplicate.
7. Place sufficient conjugate (120µL per standard/sample) in a trough and with a multichannel pipette add 100µL of conjugate to the wells already containing standard/sample. The force of the addition of the second 100µL to the first 100µL causes sufficient mixing.
8. Continue the incubation for a further 10 minutes.
9. Discard the contents of the wells into an appropriate receptacle, wash the wells by filling with PBS-Tween20 from a wash bottle or multichannel pipette. Discard the washings and repeat for a total of three washes.
10. Add 100µL of enzyme substrate (TMB) to each well and incubate for 15 minutes. Cover to avoid direct light.
11. Stop the reaction by adding 100µL stop solution. The blue color will change to yellow.
12. Read the optical density (OD) of each microwell at 450nm using an air blank or a differential filter of 630 nm.

8. 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 ng/L. The aflatoxin M_1 concentration in ng/L corresponding to the absorbance of each sample can be read from the calibration curve.

9. Specificity

The cross-reaction profile of the monoclonal antibody is as follows: M_1 100%, B_1 79%, B_2 76%, G_1 55%, M_2 33%, G_2 6%. While it is apparent that this antibody is not specific for aflatoxin M_1, the exclusive presence of aflatoxin M1 in the standards together with the fact that M_1 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 B_1, B_2, G_1, and G_2 aflatoxins. A specific antibody would not detect this potentially catastrophic occurrence.