Helica® Aflatoxin M1 ULTRA ELISA

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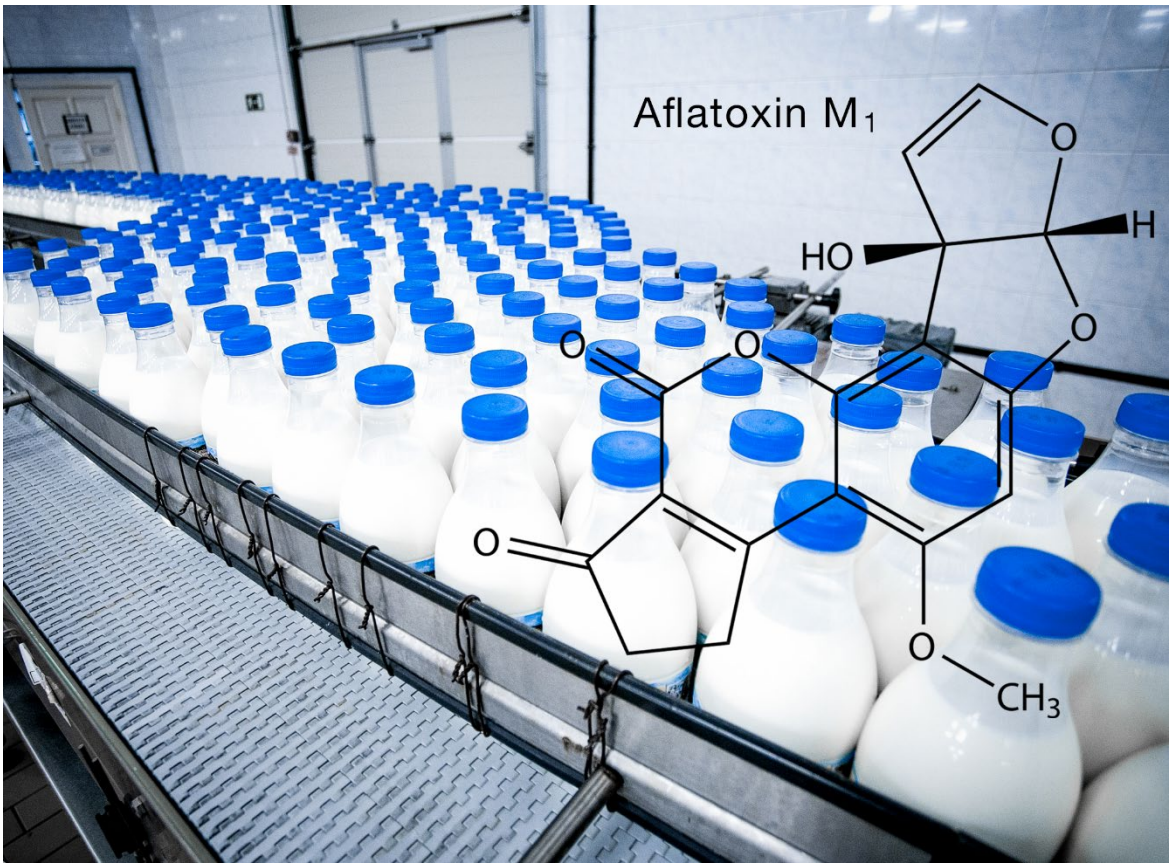

Helica® Aflatoxin M1 ULTRA ELISA

For the quantitative detection of Aflatoxin M1 in milk, skim milk powder and yogurt.

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Introduction – Aflatoxins

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 50 pg/mL (0.05 µg/L) or 50 parts per trillion (50 ppt).

Intended Use

Hygiena's Helica® Mycotoxin ELISA kits are user-friendly, cost-effective kits for the detection of mycotoxins in a wide range of commodities including animal feeds, grains, corn and animal urine, designed to protect humans and animals from dangerous side effects of mycotoxins.

The Helica Aflatoxin M1 ULTRA ELISA assay is a solid-phase competitive enzyme immunoassay for the quantitative determination of aflatoxin M1 in milk, skim milk powder and yogurt.

Data obtained from assays should not be used for human diagnostic or human treatment purposes. Assays are not approved by the United States Food and Drug Administration or any other US or non-US regulatory agency for use in human diagnostics or treatment. Assays should not be used as the sole basis for assessing the safety of products for release to consumers. The information generated is only to be used in conjunction with the user's regular quality assurance program. Not approved for clinical diagnosis. Use for research and development, quality assurance and quality control under the supervision of technically qualified persons.

Principle of the Method

The Helica Aflatoxin M1 ULTRA ELISA 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 horseradish 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 an 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 450 nm (OD450). The optical densities of the samples are compared to the ODs of the kit standards and a result is determined by interpolation from the standard curve.

Kit Contents

Package/ Number	Component	Description
1X Pouch	Antibody-coated microwell plate	96 wells (12 eight-well strips) in a microwell holder coated with a mouse anti-aflatoxin monoclonal antibody, *Ready-to-Use*.
1X Plate	Dilution plate	96 non-coated wells (12 eight-well strips) in a microwell holder, *Ready-To-Use*. (Mixing wells)
6X Vials	Standards	8.0 mL/vial of Aflatoxin M1 at the following concentrations: 0.0, 5.0, 15.0, 50.0, 150.0 and 500.0 pg/mL (ppt), *Ready-to-Use*.
1X Bottle	Conjugate	12 mL of aflatoxin conjugated to peroxidase in buffer with preservative, *Ready-to-Use*.
1X Bottle	Substrate	12 mL stabilized tetramethylbenzidine (TMB), *Ready-to-Use*.
1X Bottle	Stop solution	12 mL acidic solution, *Ready-to-Use*.
1X Pouch	PBS-T powder	PBS with 0.05% Tween® 20*, bring to 1 Liter with distilled water and store refrigerated. (Wash buffer)
1X Bottle	Milk diluent	12 mL skim milk, *Ready-to-Use*

** TWEEN® 20 is a registered trademark of CRODA International Plc.*

Materials Required But Not Provided

- Microtubes
- Single or multichannel pipettors with tips: 10 µL, 100 µL, 200 µL and 1000 µL
- Timer
- Wash bottle
- Absorbent paper towels
- Microplate reader with 450 nm filter
- Centrifuge (and tubes)
- Yogurt diluent for yogurt samples (Cat# 937YOG001)

Storage and Shelf Life

- Store reagents at 2 to 8 °C. Do not freeze.
- Reagents should be used by the expiration date stamped on the individual labels.
- HRP-labeled conjugate and TMB-substrates are photosensitive and are packaged in a light-protective opaque bottle. Store in the dark and return to storage after use.

Precautions and Waste Disposal

General Precautions:

- Bring all reagents to room temperature (19 to 25 °C) before use.
- Do not interchange reagents between kits of different lot numbers.
- Do not use solutions if cloudy or precipitate is present.
- Do not return unused reagents to their original bottles. The assay procedure details volumes required.
- Adhere to all time and temperature conditions stated in the procedure.
- During the sample extraction, avoid cross-contamination.
- Devices, such as a blender, must be cleaned after each sample preparation.
- Samples tested should have a pH of 7.0 (± 1.0). Excessive alkaline or acidic conditions may affect the test results.

Safety Precautions:

Mycotoxins (aflatoxins, trichothecenes and others) are well-known human carcinogens and are considered highly toxic. Because mycotoxins can cause human illness, appropriate safety precautions must be taken and personal protective equipment worn when handling samples, reagents, glassware and other supplies and equipment that potentially could be contaminated with mycotoxins.

- Before using this assay, please review the Safety Data Sheet(s) available at www.hygiena.com.
- Refer to your site practices for safe handling of materials.
- It is strongly advised that protective gloves, a lab coat and safety glasses be worn at all times while handling mycotoxin kits and their respective components. Consider all materials, containers and devices that are exposed to samples or standards to be contaminated with mycotoxins.
- Never pipette reagents or samples by mouth.
- Standards are flammable. Caution should be taken in the use and storage of these reagents.
- The stop solution contains sulfuric acid, which is corrosive. Please refer to the SDS. Do not allow to contact skin or eyes. If exposed, flush with water.

Disposal:

Decontaminate materials and dispose of waste per your site practices and as required by local regulations. Do not dispose of these materials down the drain. Please note that there is a potential for mycotoxin contamination in or on any of the kit components provided.

- Dispose of all materials, containers and devices in the appropriate receptacle after use.
  Before conducting the assay, prepare a waste container to act as a receptacle for your kit waste.
  Eject contaminated pipette tips and discard all other related materials into this container.
- Once the assay is completed, the container should be treated with a sufficient amount of 5 - 6% sodium hypochlorite (NaOCl) to saturate the contents of the container (approximately 1/10th the volume of the container). The NaOCl will denature the mycotoxins and neutralize the waste, making it safe for disposal. Invert the container several times to thoroughly coat all waste.
- In the case of an accidental toxin spill, treat the spill surface with 5 - 6% NaOCl for a minimum of 10 minutes, followed by 5% aqueous acetone. Wipe dry with absorbent paper towels.

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 a 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 15,000 x g 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. Transfer 1 mL of milk into a microcentrifuge tube.
2. Centrifuge at 15,000 x g for 5 minutes to induce separation of the upper fatty layer.
3. Remove the upper fatty layer by aspiration and transfer the clean mid-plasma layer to a microtube for the assay.

Milk Powder

1. Reconstitute milk powders according to the manufacturer’s instructions and treat the reconstituted product as above.
2. Transfer into a microtube for the assay.

Yogurt

1. For testing yogurt, a yogurt diluent must be used and is sold separately. (Cat# 937YOG001; Please contact us to purchase.)
2. Weigh out 1 g of yogurt into a clean tube.
3. Add 3 mL of yogurt diluent into the tube.
4. Vortex the tube for approximately 30 seconds until the mixture is homogenized.
5. Transfer to a microtube for the assay.
6. The measured value of Aflatoxin M1 from the sample must be multiplied by 4 to account for the sample dilution.

Assay Procedure

1. Bring the reagents to room temperature before use. Prepare wash buffer by reconstituting the contents of the PBS-T powder packet by washing out the contents with a gentle stream of distilled or deionized water into a 1-Liter container. Fill to 1 Liter with distilled water and store refrigerated when not in use.
2. Place one mixing well in a microwell holder for each standard and sample to be tested. Place twice the number of antibody-coated microtiter wells in another microwell holder. If running a single well, adjust volumes accordingly. Return unused wells to the foil pouch with desiccant and reseal.
3. Mix each reagent by swirling the reagent bottle prior to use.
4. Aliquot 1.2 mL of standards and sample into microtubes. If running singlets, scale the volume down accordingly.

5. Transfer 200 µL aliquots of standards and samples from the microtubes into the antibody-coated wells in duplicate. (It is recommended to use a multichannel pipettor for the liquid transfer). Incubate for 20 minutes.
   Note: The microtubes contain enough solution to run each standard and/or sample in duplicate (recommended). If running each standard or sample in singlets or if more replicates are needed, the volumes of assay diluent and sample/ standard should be scaled accordingly.
6. Decant the contents from microwells into a discard basin. Wash the microwells by filling each with PBS-T wash buffer, then decanting the wash into a discard basin. Repeat wash for a total of 3 washes.
7. Tap the microwells (face down) on a layer of absorbent towels to remove residual wash buffer.
8. Transfer 200 µL aliquots of standards and samples again. Incubate for 20 minutes again (the second incubation).
9. During the second incubation, dispense 150 µL of standard or sample into each mixing well, and add 150 µL of the conjugate to each well. Mix by priming pipettor at least 3 times. If running singlets, scale the volume down accordingly.
   Note: The operator must record the location of each Standard and Sample throughout the test.
10. After the second incubation, wash the plate by repeating step 8.
11. Transfer 100 µL of the conjugate mixture from each mixing well (step 8) to a corresponding antibody-coated well. Incubate at ambient temperature for 20 minutes. Cover to avoid direct light.
12. Add 100 µL of enzyme substrate (TMB) to each well and incubate for 10 minutes. Cover to avoid direct light.
13. Stop the reaction by adding 100 µL stop solution. The blue color will change to yellow.
14. Read the optical density (OD) of each microwell with a microplate reader at 450 nm using an air blank or a differential filter of 630 nm.

Interpretation of Results

Construct a dose-response curve using 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.

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

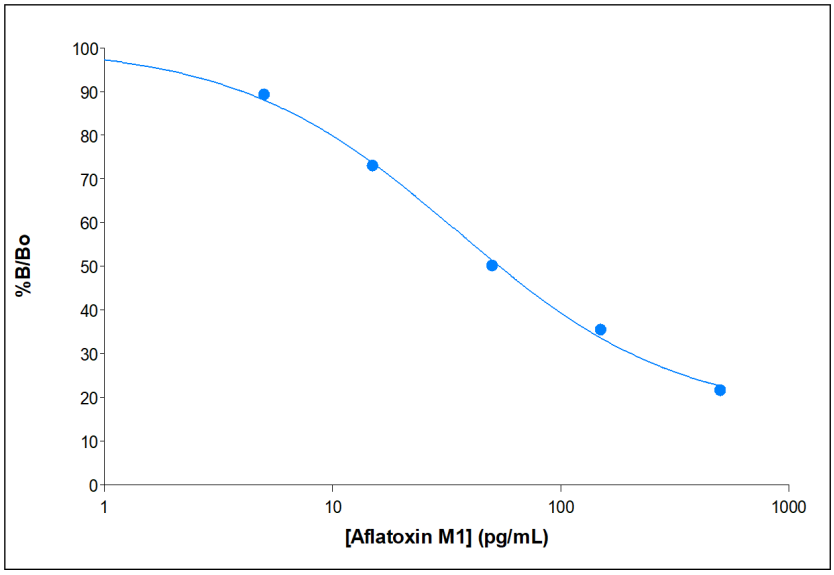
The values calculated for the standards are entered in a system of coordinates on 4-parameter graph paper against the Aflatoxin M1 concentration in pg/mL. The Aflatoxin M1 concentration in pg/mL corresponding to the absorbance of each sample can be read from the calibration curve.

(To obtain the Aflatoxin M1 concentration in pg/mL 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 4 for yogurt samples).

Assay Characteristics

Data from nine (9) consecutive standard curves gave the following results.

Aflatoxin M1 (pg/mL)	% B/B$_0$	CV (%)
0	100	0.45
5	89	0.04
15	71	2.14
50	44	1.62
150	29	0.22
500	17	2.60



Recovery and Reproducibility

Recovery of 50 ppt spiked into milk is as follows (average of three sample for each sample type and level):

Type of Dairy Sample	Spike (ppt)	n	% Recovery	CV (%)
Raw milk	5	3	92	12.82
	50	3	115	1.82
	200	3	95	0.61
Homogenized milk	5	3	101	7.76
	50	3	115	2.43
	200	3	101	5.45
Skim milk powder	5	3	80	8.74
	50	3	109	4.12
	200	3	99	2.93
Yogurt	20	3	99	14.60
	50	3	112	11.40
	200	3	110	6.47

Cross-reactivity

The table below shows the cross-reactivity of the antibody to each aflatoxin.

Aflatoxin Subtype	Cross-reactivity (%)
Aflatoxin M1	100
Aflatoxin M2	<0.1
Aflatoxin B1	<0.1
Aflatoxin B2	<0.1
Aflatoxin G1	<0.1
Aflatoxin G2	<0.1

Technical Assistance

For questions or comments, please contact your local distributor. You can also email techsupport@hygiena.com, visit our Contact Us page for regional phone numbers or request technical support at https://www.hygiena.com/hygiena/technical-support-request.html.