TOTAL AFLATOXIN ASSAY – LOW MATRIX

Cat.No. 981AFL01LM-96

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, cottonseeds 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. Symptoms of toxicity in animals range from death to chronic diseases, reproductive interference, immune suppression, and decreased milk and egg production. Most controlling government agencies worldwide have regulations regarding the amount of aflatoxins allowable in human and animal foodstuffs. Accurate and rapid determination of the presence of aflatoxin in commodities is of paramount importance.

INTENDED USE

The HELICA Low Matrix Total Aflatoxin Assay is a competitive enzyme-linked immunoassay for the quantitative detection of aflatoxin B_1, B_2, G_1 and G_2 in grains, nuts, cotton seed, cereals and all commodities which are difficult to measure due to high matrix effect such as silage and most spices.

FIELD OF USE

Data obtained from Helica™ assays should not be used for human diagnostic or human treatment purpose. Assays are not approved by the United States Food and Drug Administration or any other U.S. or non-U.S. regulatory agency for use in human diagnostics or treatment. Helica™ 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 supervision of technically qualified persons.

ASSAY PRINCIPLE

The HELICA Low Matrix Total Aflatoxin Assay is a solid phase competitive inhibition enzyme immunoassay. An aflatoxin specific antibody optimized to cross react with all four subtypes of aflatoxin (see cross-reactivity information), is coated to a polystyrene microwell. Toxins are extracted from a ground sample with 50% methanol, 80% methanol or 80% acetonitrile and after dilution, added to the appropriate well. If aflatoxin 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 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 in the standard or sample. Therefore, as the concentration of aflatoxin 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 ODs of the kit standards and a result is determined by interpolation from the standard curve.

REAGENTS PROVIDED

1X Pouch	**Antibody Coated Microwell plate**		96 wells (12 X 8 well strips) in a microwell holder coated with a mouse anti-aflatoxin monoclonal antibody, *Ready-to-Use.*
1X Plate	**Mixing Wells**	Green	96 non-coated wells (12 X 8 well strips) in a microwell holder, *Ready-to-Use.*
6X Vials	**Aflatoxin Standards**	Black Cap	1.5mL/vial of aflatoxin at the following concentrations: 0.0, 0.02, 0.05, 0.1, 0.2, and 0.4ng/mL in 50% methanol, *Ready-to-Use.*
1X Bottle	**Aflatoxin HRP-Conjugate**	Green Cap	12mL of aflatoxin B_1 conjugated to peroxidase in buffer with preservative, *Ready-to-Use.*
2X Bottles	**Sample Diluent**	Brown Cap	2 x 12mL proprietary sample diluent, *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**		PBS WITH 0.05% Tween20, bring to 1 liter with distilled water and store refrigerated.

****A modified assay diluent for testing infant and toddler milk formulas is supplied separately. Please ask your supplier for a free bottle of modified assay diluent if needed. Shake the bottle well before using.**

MATERIALS REQUIRED BUT NOT PROVIDED

Extraction Procedure
- Grinder sufficient to render sample to particle size of fine instant coffee
- Collection Container: Minimum 250mL capacity
- Balance: 20g measuring capability
- Graduated cylinder: 250mL
- Methanol or acetonitrile: 50-200mL reagent grade per sample.
- Distilled or deionized water: 20-50mL per sample
- Filter Paper: Whatman #1 or equivalent
- Filter Funnel
- Centrifuge

Assay Procedure
- Pipettor with tips: 100μL and 200μL
- Timer
- Wash bottle
- Dilution tubes
- Absorbent paper towels
- Microplate reader with 450nm filter

PRECAUTIONS
1. Bring all reagents to room temperature (19º - 27ºC) before use.
2. Store reagents at 2°-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. Wear protective gloves and safety glasses when using this kit.
8. Dispose of all materials, containers and devices in the appropriate receptacle after use.
9. HRP-labeled conjugate and TMB - substrates are photosensitive and are packaged in a protective opaque bottle. Store in the dark and return to storage after use.

KIT SAFETY AND WASTE DISPOSAL INSTRUCTIONS

Mycotoxins (aflatoxins, trichothecenes, and others) are well known carcinogens in humans and are thus considered highly toxic. 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. It is strongly advised that gloves, a lab coat, and safety glasses be worn at all times while handling mycotoxin kits and their respective components.

1. Before conducting the assay, prepare a waste container to act as a receptacle for your kit waste. Eject contaminated pipette tips and all other related materials into this container.
2. 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). 5-6% NaOCl will denature the mycotoxins and neutralize the waste, making it safe for disposal. Invert the container several times to thoroughly coat all waste.
3. 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.

EXTRACTION PROCEDURE

Note: The sample must be collected according to the appropriate established sampling techniques

Corn, Wheat, Hay, Snaplage, Paprika, Pistachio, and Peanut

1. Grind a representative sample to the particle size of fine instant coffee (50% passes through a 20 mesh screen).
2. Prepare extraction solvent (80% methanol or 80% acetonitrile) by adding 20mL of distilled or deionized water to 80mL of methanol or acetonitrile for each sample to be tested.
3. Transfer 100mL of extraction solvent to a container and add 20g of the ground sample.
   Note: The ratio of sample to extraction solvent is 1:5(w/v).
4. Mix by shaking in a sealed container or in a blender for a minimum of 2 minutes.
5. Allow the particulate matter to settle, then filter 5 - 10mL of the extract through a Whatman #1 filter paper (or equivalent) and collect the filtrate to be tested.
6. Dilute an aliquot of the extract 1:10 with reconstituted wash buffer.
7. The sample is now ready. The standards require no pre-dilution before use.
8. Final dilution for use in calculation = 1:50

Soy sauce

1. Prepare extraction solvent (80% acetonitrile) by adding 20mL of distilled or deionized water to 80mL of acetonitrile for each sample to be tested.
2. Transfer 100mL of 80% acetonitrile to a container and add 20mL of sample.
   Note: The ratio of sample to extraction solvent is 1:5 (v/v).
3. Mix by shaking in a sealed container for a minimum of 5 minutes.
4. Allow the acetonitrile and soy sauce layers to separate. Alternatively, centrifuge a portion of the sample at 3,500rpm for 5 minutes to speed the separation. Collect the upper layer containing the aflatoxin to be tested.
5. Dilute an aliquot of the extract 1:10 with reconstituted wash buffer.

6. The sample is now ready. The standards require no pre-dilution before use.

7. Final dilution for use in calculation = 1:50

Soy bean, Chili, Cilantro, and Coriander

1. Grind a representative sample to the particle size of fine instant coffee (50% passes through a 20 mesh screen).
2. Prepare extraction solvent (80% acetonitrile) by adding 20mL of distilled or deionized water to 80mL of acetonitrile for each sample to be tested.
3. Transfer 100mL of 80% acetonitrile to a container and add 20g of the ground sample.
   Note: The ratio of sample to extraction solvent is 1:5 (w/v).
4. Mix by shaking in a sealed container for a minimum of 5 minutes.
5. Centrifuge the sample at 3,500rpm for 5 minutes. Alternatively, pass a 5-10mL portion of the sample through a filter and collect the filtrate to be tested.
6. Dilute an aliquot of the extract 1:10 with reconstituted wash buffer.
7. The sample is now ready. The standards require no pre-dilution before use.
8. Final dilution for use in calculation = 1:50

Corn oil and Peanut oil

1. Prepare extraction solvent (80% acetonitrile) by adding 40mL of distilled or deionized water to 160mL of acetonitrile for each sample to be tested.
2. Transfer 200mL of 80% acetonitrile to a container and add 10mL of sample.
   Note: The ratio of sample to extraction solvent is 1:20 (v/v).
3. Mix by shaking in a sealed container for a minimum of 30 minutes.
4. Allow the acetonitrile and oil layers to separate. Alternatively, centrifuge a portion of the sample at 3,500rpm for 5 minutes to speed the separation. Collect the upper layer containing the aflatoxin to be tested.
5. Dilute an aliquot of the extract 1:10 with reconstituted wash buffer.
6. The sample is now ready. The standards require no pre-dilution before use.
7. Final dilution for use in calculation = 1:200

Safflower oil, Sesame oil, and Vegetable oil

1. Prepare extraction solvent (80% acetonitrile) by adding 20mL of distilled or deionized water to 80mL of acetonitrile for each sample to be tested.
2. Transfer 100mL of 80% acetonitrile to a container and add 10mL of sample.
   Note: The ratio of sample to extraction solvent is 1:10 (v/v).
3. Mix by shaking in a sealed container for a minimum of 30 minutes.
4. Allow the acetonitrile and oil layer to separate. Alternatively, centrifuge a portion of the sample at 3,500rpm for 5 minutes to speed the separation. Collect the upper layer containing the aflatoxin to be tested.
5. Dilute an aliquot of the extract 1:10 with reconstituted wash buffer.
6. The sample is now ready. The standards require no pre-dilution before use.
7. Final dilution for use in calculation = 1:100

Infant and Toddler milk formulas

1. Prepare extraction solvent (50% methanol) by adding 50mL of distilled or deionized water to 50mL of pure methanol.
2. Transfer 100mL of the 50% methanol to a container and add 20g of sample.
   Note: The ratio of sample to extraction solvent is a 1:5 dilution (w/v).
3. Mix by shaking in a sealed container for a minimum of 10 minutes.
4. Centrifuge the sample at 3,500rpm for 5 minutes to pellet the particulate matter.
5. Collect the supernatant and proceed to the assay procedures. No further dilution of the sample into wash buffer is necessary.

Note: Depending on the formulation, some infant formulas will contain a floating fatty layer that must be aspirated. Use the lower plasma layer for the analysis.

6. Final dilution for use in calculation = 1:5

Toddler rice cereal

1. Grind a representative sample to a fine particle size comparable to powdered sugar. The sample does not need to be passed through a mesh screen.
2. Prepare extraction solvent (50% methanol) by adding 50mL of distilled or deionized water to 50mL of pure methanol.
3. Transfer 100mL of 50% methanol to a container and add 20g of the ground sample.

   Note: The ratio of sample to extraction solvent is a 1:5 dilution (w/v).
4. Mix by shaking in a sealed container for a minimum of 10 minutes.
5. Centrifuge the sample at 3,500rpm for 5 minutes to pellet the particulate matter.
6. Collect the supernatant and proceed to the assay procedures. No further dilution of the sample into wash buffer is necessary.
7. Final dilution for use in calculation = 1:5

Animal feed

1. Grind a representative sample to the particle size of fine instant coffee (50% passes through a 20 mesh screen)
2. Prepare extraction solvent (80% acetonitrile) by adding 40mL of distilled or deionized water to 160mL of acetonitrile for each sample to be tested.
3. Transfer 200mL of 80% acetonitrile to a container and add 2g of the ground sample.

   Note: The ratio of sample to extraction solvent is a 1:100 dilution (w/v).
4. Mix by shaking in a sealed container for a minimum of 10 minutes.
5. Centrifuge the sample at 3,500rpm for 5 minutes to pellet the particulate matter.
6. Collect the supernatant containing aflatoxin for analysis.
7. Dilute an aliquot of the extract 1:10 in reconstituted wash buffer.
8. Final dilution for use in calculation = 1:1000

Potable Water

This assay may be used for detecting aflatoxin in potable water. In this case, use the sample as is without any extraction or dilution.

ASSAY PROCEDURE

1. Bring all the reagents to room temperature before use. Reconstitute the PBS-Tween packet by washing out the contents with a gentle stream of distilled water into a 1-Liter container. Q.S. 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 an equal number of Antibody Coated Microtiter Wells in another microwell holder.
3. Dispense 200µL of the Sample Diluent into each mixing well.
   Note: For infant or toddler milk formula samples, use the modified assay diluent supplied separately (Cat#986BAF01LM-F). Please ask your supplier for a free bottle of assay diluent. Shake the bottle well before using. Use the modified assay diluent for the standards ONLY. For the unknown samples, use the sample diluent already supplied with this kit.
4. Using a new pipette tip for each, add 100µL of each standard and prepared sample to the appropriate mixing well containing diluent. Mix by priming pipettor at least 3 times.
   Note: Operator must record the location of each Standard and Sample throughout test.
5. Using a new pipette tip for each, transfer 100µL of contents from each mixing well to a corresponding Antibody Coated Microtiter Well. Incubate at room temperature for 30 minutes. The mixing wells contain enough solution to run each standard and/or sample in duplicate if so desired.
6. Decant the contents from microwells into a discard basin. Wash the microwells by filling each with PBS-Tween 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. Add 100µL of Aflatoxin HRP-conjugate to each antibody coated well and incubate at room temperature for 30 minutes. Cover to avoid direct light.
9. Repeat steps 6 and 7.
10. Measure the required volume of Substrate Solution (1 mL/strip or 120µL/well) and place in a separate container. Add 100µL to each microwell. Incubate at room temperature for 10 minutes. Cover to avoid direct light.
11. Measure the required volume of Stop Solution (1 mL/strip or 120µL/well) and place in a separate container. Add 100µL in the same sequence and at the same pace as the Substrate Solution was added.
12. Read the optical density (OD) of each microwell with a microtiter plate reader using a 450nm filter. Record the optical density (OD) of each microwell.
13. Setting the zero standard as 100% binding (Bo), calculate % binding (%B) for each standard and sample as a percentage of the zero binding (%B/Bo).

INTERPRETATION OF RESULTS (SEE EXTRACTION PROCEDURE ON PAGES 3 or 4 FOR SPECIFIC APPLICATIONS)

Construct a dose-response curve using either the unmodified OD values or the OD values expressed as a percentage (%B/Bo) of the OD of the zero (0.0) standard against the aflatoxin content of the standard. Unknowns are measured by interpolation from the standard curve.

The information contained on the label of each standard vial refers to the contents of that vial. However, the sample has been diluted at a 5:1, 10:1, 20:1, or 100:1 ratio by extraction solvent as instructed in the EXTRACTION PROCEDURE and also 10:1 in wash buffer (except no dilution for baby, toddler formulas and cereal) and so the level of aflatoxin shown by the standard must be multiplied by 5, 50, 100, 200, or 1000 in order to indicate the ng per gram (ppb) of the commodity as follows:

	Infant and toddler milk formulas and toddler rice cereal	Corn, wheat, silage, peanut, soy sauce, soy bean	Safflower oil, sesame oil, and vegetable oil	Corn oil and peanut oil	Animal feed
standard ng/mL	**commodity (ppb) 1:5**	**commodity (ppb) 1:50**	**commodity (ppb) 1:100**	**commodity (ppb) 1:200**	**commodity (ppb) 1:1000**
0	0	0	0	0	0
0.02	0.1	1	2	4	20
0.05	0.25	2.5	5	10	50
0.1	0.5	5	10	20	100
0.2	1	10	20	40	200
0.4	2	20	40	80	400

The sample dilution results in a standard curve: 0.1 – 2ppb, 1 – 20ppb, 2 – 40ppb, 4 – 80ppb, or 20 – 400ppb depending on the dilution factors (see EXTRACTION PROCEDURE section). If a sample contains aflatoxin at greater concentration than the highest standard, it should be diluted appropriately in extraction solvent and retested. The extra dilution step should be taken into consideration when expressing the final result.

In the case of potable water there is no pre-dilution, so it is measured with a sensitivity equal to the lowest standard which is 20 parts per trillion (ppt).

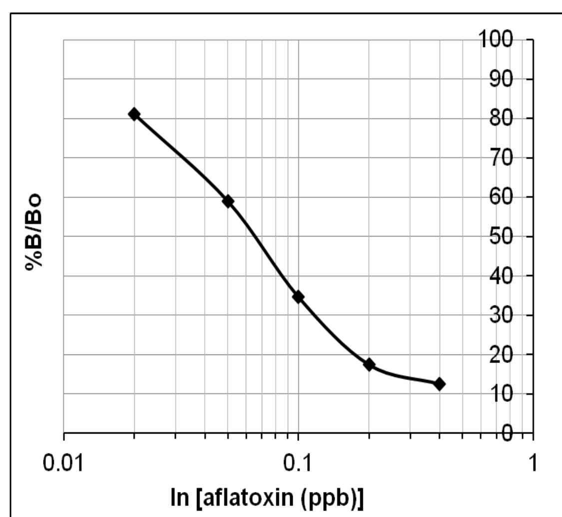
ASSAY CHARACTERISTICS

Data from ten consecutive standard curves using wheat as an example gave the following results:

Standard (ng/mL)	Concentration in commodity (1:50)	%B/B_0	%CV

0	0	100	-
0.02	1	81	1.3
0.05	2.5	59	2
0.1	5	34.7	5.5
0.2	10	17.5	4.9
0.4	20	12.6	7.6

The below figure is a representative standard curve for aflatoxin based on the above data table.



As an example of a high matrix effect commodity, thirteen silage samples, 5 corn, 2 wheat, 3 hay and 3 snaplage which had measured less than 1ppb for B_1, B_2, G_1 and G_2 by HPLC were extracted with either 80% methanol or 80% acetonitrile.

Following extraction with 80% methanol, 12/13 measured less than 1ppb, with a single wheat silage sample measuring 1.2ppb. After extraction with 80% acetonitrile, 8/12 measured less than 1ppb with 5 samples averaging 1.5ppb. No sample measured more than 2ppb.

Recoveries of a 5ng/gm spike into four of the silage samples were as follows:

	Acetonitrile extract			Methanol extract	
	ppb	% Recovery		ppb	% Recovery
Spike	**4.8**	**100**		**5.1**	**100**
Corn	4.1	85		2.5	49
Wheat	4.8	100		2.7	53
Hay	4.6	96		2.7	53
Snaplage	4.6	96		2.9	57

In a similar experiment, extraction of paprika, pistachio, and peanut by either methanol or acetonitrile was less than 1ppb and after a 5ppb spike recoveries were 96%, 93%, and 67%, respectively, for acetonitrile and 67%, 69% and 58% for methanol.

Recoveries of 5ppb spiked into three soy sauce or soy bean samples extracted with 80% acetonitrile were as follows based on four independent experiments:

Type of Commodity	ppb	% Recovery
Soy sauce	5.1	102
Soy bean	4.6	92

Recoveries of 0.5ppb spiked into infant or toddler food samples extracted with 50% methanol were as follows based on four independent experiments:

Type of Commodity	ppb	% Recovery
Infant milk formula	0.49	98
Toddler milk formula	0.48	95
Toddler rice cereal	0.44	88

Recoveries of 5ppb spiked into chili powder, ground coriander seed, and ground cilantro seed extracted with 80% acetonitrile were as follows based on four independent experiments:

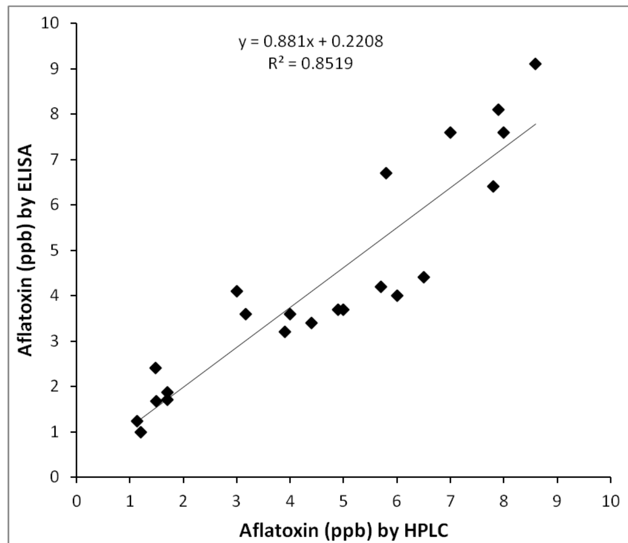
Type of Commodity	ppb	% Recovery
Chili powder	4.8	95.3
Cilantro seed	4.8	95.0
Coriander seed	5.1	101.3

Recoveries of 20ppb or 10ppb spiked into oil samples extracted with 80% acetonitrile were as follows based on three independent experiments:

Type of Commodity	ppb	% Recovery
Corn oil (20ppb)	17.5	87
Peanut oil (20ppb)	17.1	85
Safflower oil (10ppb)	9.3	93
Sesame oil (10ppb)	7.8	78
Vegetable oil (10ppb)	9.3	93

Acetonitrile is the preferred extraction solvent but methanol may be used if its extraction efficiency is taken into account.

Correlation studies were also completed to compare the performance between HELICA'S Low Matrix Total Aflatoxin Assay and HPLC determination. The figure below shows that there is an excellent correlation between HELICA's Low Matrix Total Aflatoxin Assay and HPLC over a range of <0.5 to >8ppb on 23 chili samples.



The graph above illustrates the excellent correlation displayed between HELICA's ELISA and HPLC analysis of chili samples (n=23) containing <0.5 to 8ppb of aflatoxin.

CROSS-REACTIONS

The assay will cross-react with aflatoxin analogues as follows:
B_1 - 100%, B_2 - 77%, G_1 - 64%, G_2 - 25%

REFERENCES

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3. Williams JH, Phillips TD, Jolly PE, Stiles JK, Jolly CM, and Aggarwal D (2004). Human aflatoxicosis in developing countries: a review of toxicology, exposure, potential health consequences, and interventions. Am J Clin Nutr. 80(5): 1106-1122.

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