



## Helica® Total Aflatoxin Low Matrix ELISA

Product Number – KIT5006 (981AFL01LM – 96)





## Total Aflatoxin Low Matrix ELISA

*For the quantitative detection of aflatoxin B1, B2, G1 and G2 in grains, nuts, cotton seeds, cereals and all commodities which are difficult to measure due to high matrix effects such as silage and most spices.*

### Table of Contents

Introduction – Aflatoxins .....	3
Intended Use.....	3
Principle of the Method .....	3
Kit Contents.....	4
Materials Required But Not Provided.....	4
Storage and Shelf Life .....	5
Precautions and Waste Disposal.....	5
Preparation of Samples.....	6
Assay Procedure.....	8
Interpretation of Results.....	9
Assay Characteristics.....	10
Cross Reactivity.....	12
References .....	12
Technical Assistance .....	12



## 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, cottonseeds and other commodities associated with human food or animal feeds. Crops may be contaminated by one or more of the four 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 high.

Aflatoxins have been implicated in human health disorders including hepatocellular carcinoma, aflatoxicosis, Reye's syndrome and chronic hepatitis. Animals are exposed to aflatoxins by consuming 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

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 Total Aflatoxin Low Matrix ELISA assay is a competitive enzyme-linked immunoassay for the quantitative detection of aflatoxin B1 in nuts, cotton seeds, cereals and all commodities which are difficult to measure due to high matrix effects such as silage and most spices.

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 Total Aflatoxin Low Matrix ELISA 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 on page 12), 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 450 nm (OD450). The optical densities of the samples are compared to the ODs of the kit standards and results are 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 antibody, <i>Ready-to-Use</i> .
1X Plate	Dilution plate	96 non-coated wells (12 eight-well strips) in a microwell holder, <i>Ready-to-Use</i> . (Mixing wells)
6X Vials	Standards	1.5 mL/vial of Aflatoxin at the following concentrations: 0.0, 0.02, 0.05, 0.1, 0.2 and 0.4 ng/mL in 50% methanol, <i>Ready-to-Use</i> .
1X Bottle	Conjugate	12 mL of Aflatoxin B1 conjugated to peroxidase in buffer with preservative, <i>Ready-to-Use</i> .
2X Bottles	Assay diluent	2 x 12 mL proprietary sample diluent, <i>Ready-to-Use</i> .
1X Bottle	Substrate	12 mL stabilized tetramethylbenzidine (TMB), <i>Ready-to-Use</i> .
1X Bottle	Stop solution	12 mL acidic solution, <i>Ready-to-Use</i> .
1X Pouch	PBS-T powder	PBS with 0.05% Tween® 20*, bring to 1 Liter with distilled water and store refrigerated. (Wash buffer)

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

## Materials Required But Not Provided

- Grinder sufficient to render sample to a particle size of fine instant coffee
- Collection tube: Minimum 250 mL capacity
- Balance: up to 20 g measuring capability
- Graduated cylinder: 250 mL
- Methanol or acetonitrile, reagent grade: 50 – 200 mL per sample
- Distilled or deionized water: 20 – 50 mL per sample
- Filter paper: Whatman #1 or equivalent
- Filter funnel
- Centrifuge
- Pipettor with tips: 100 µL and 200 µL
- Dilution tubes
- Timer
- Wash bottle
- Absorbent paper towels
- Microplate reader with 450 nm filter



## 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 ( $\pm$  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](http://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

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

### Extraction Procedure

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

1. Grind a representative sample to the particle size of fine instant coffee (95% passes through a 20-mesh screen).
2. Prepare the extraction solution (80% methanol or 80% acetonitrile) by adding 20 mL of distilled or deionized water to 80 mL of methanol or acetonitrile (reagent grade) for each sample to be tested.
3. Weigh out a 20 g ground portion of the sample and add 100 mL of the Extraction Solvent (80%).  
*Note: The ratio of sample to extraction solvent is 1:5 (w/v).*
4. Mix by shaking in a sealed container or a blender for a minimum of 2 minutes.
5. Allow the particulate matter to settle, then filter 5 – 10 mL 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 PBS-T.
7. The sample is now ready for testing.
8. The final dilution for use in calculations is **1:50**.

#### Soy Sauce

1. Prepare the extraction solution (80% acetonitrile) by adding 20 mL of distilled or deionized water to 80 mL of acetonitrile (reagent grade) for each sample to be tested.
2. Place 20 mL of the sample into a container and add 100 mL of the Extraction Solvent (80% acetonitrile).  
*Note: The ratio of sample to extraction solvent is 1:5 (v/v).*
3. Mix by shaking in a sealed container or a blender for a minimum of 5 minutes.
4. Allow the acetonitrile and soy sauce layers to separate. Alternatively, centrifuge a portion of the sample at 3,500 rpm 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 PBS-T.
6. The sample is now ready for testing.
7. The final dilution for use in calculations is **1:50**.

#### Soybean, Chili, Cilantro and Coriander

1. Grind a representative sample to the particle size of fine instant coffee (95% passes through a 20-mesh screen).
2. Prepare extraction solvent (80% acetonitrile) by adding 20 mL of distilled or deionized water to 80 mL of acetonitrile for each sample to be tested.
3. Transfer 100 mL of 80% acetonitrile to a container and add 20 g 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 3,500 rpm for 5 minutes. Alternatively, pass a 5-10 mL 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 PBS-T.
7. The sample is now ready.
8. The final dilution for use in calculations is **1:50**.



### Corn Oil and Peanut Oil

1. Prepare the extraction solution (80% acetonitrile) by adding 40 mL of distilled or deionized water to 160 mL of methanol or acetonitrile (reagent grade) for each sample to be tested.
2. Place 10 mL of the sample into a container and add 200 mL of the Extraction Solvent (80% acetonitrile).  
*Note: The ratio of sample to extraction solvent is 1:20 (v/v).*
3. Mix by shaking in a sealed container or a blender for a minimum of 30 minutes.
4. Allow the acetonitrile and oil layers to separate. Alternatively, centrifuge a portion of the sample at 3,500 rpm 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 PBS-T.
6. The sample is now ready for testing.
7. The final dilution for use in calculations is **1:200**.

### Safflower Oil, Sesame Oil and Vegetable Oil

1. Prepare the extraction solution (80% acetonitrile) by adding 40 mL of distilled or deionized water to 160 mL of methanol or acetonitrile (reagent grade) for each sample to be tested.
2. Place 10 mL of the sample into a container and add 100 mL of the Extraction Solvent (80%).  
*Note: The ratio of sample to extraction solvent is 1:10 (v/v).*
3. Mix by shaking in a sealed container or a blender for a minimum of 30 minutes.
4. Allow the acetonitrile and oil layers to separate. Alternatively, centrifuge a portion of the sample at 3,500 rpm 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 PBS-T.
6. The sample is now ready for testing.
7. The final dilution for use in calculations is **1:100**.

### Infant and Toddler Milk Formulas

1. Prepare the extraction solution (50% methanol) by adding 50 mL of distilled or deionized water to 50 mL of methanol (reagent grade) for each sample to be tested.
2. Place 20 g of the sample into a container and add 100 mL of the Extraction Solvent (50% methanol).  
*Note: The ratio of sample to extraction solvent is 1:5 (w/v).*
3. Mix by shaking in a sealed container or a blender for a minimum of 10 minutes.
4. Centrifuge the sample at 3,500 rpm for 5 minutes to pellet the particulate matter.
5. Collect the supernatant containing the aflatoxin to be tested and proceed to the assay procedures. No further dilution of the sample into the 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. The final dilution for use in calculations is **1:5**.

### Toddler Rice Cereal

1. Grind a representative sample to the particle size of powdered sugar. The sample does not need to be passed through a mesh screen.
2. Prepare extraction solvent (50% methanol) by adding 50 mL of distilled or deionized water to 50 mL of methanol for each sample to be tested.
3. Transfer 100 mL of 50% methanol to a container and add 20 g 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 10 minutes.
5. Centrifuge the sample at 3,500 rpm for 5 minutes to pellet the particulate matter.



6. Collect the supernatant and proceed to the assay procedure. No further dilution of the sample into the wash buffer is necessary.
7. The final dilution for use in calculations is **1:5**.

## Animal Feed

1. Grind a representative sample to the particle size of fine instant coffee (95% passes through a 20-mesh screen).
2. Prepare extraction solvent (80% acetonitrile) by adding 40 mL of distilled or deionized water to 160 mL of acetonitrile for each sample to be tested.
3. Transfer 200 mL of 80% acetonitrile to a container and add 2 g of the ground sample.  
*Note: The ratio of sample to extraction solvent is 1:100 (w/v).*
4. Mix by shaking in a sealed container for a minimum of 10 minutes.
5. Centrifuge the sample at 3,500 rpm 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 PBS-T.
8. The final dilution for use in calculations is **1:1000**.

## Potable Water

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

## Assay Procedure

1. Bring all the reagents to room temperature before use. Prepare wash buffer by reconstituting 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 or deionized 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. If running 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. Dispense 200  $\mu$ L of the Assay Diluent into each mixing well.  
*Note: For infant or toddler milk formula samples, use the modified assay diluent supplied separately (Cat# 986BAF01LM-F). (Shake the bottle well before use. Use the modified assay diluent for the standards ONLY). For unknown samples, use the sample diluent already supplied with this kit.*
5. Using a new pipette tip for each, add 100  $\mu$ L of each standard and prepared sample to the appropriate mixing well containing diluent. Mix by priming pipettor at least three (3) times.  
*Note: The operator must record the location of each standard and sample throughout the test.*
6. Using a new pipette tip for each, transfer 100  $\mu$ L of contents from each mixing well to a corresponding antibody-coated microtiter well. Incubate at room temperature for 30 minutes.  
*Note: The mixing wells 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.*
7. 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 three (3) washes.
8. Tap the microwells (face down) on a layer of absorbent towels to remove residual wash buffer.
9. Add 100  $\mu$ L of aflatoxin HRP-conjugate to each antibody-coated well and incubate at room temperature for 30 minutes. Cover to avoid direct light.
10. Repeat steps 6 and 7.





11. Measure the required volume of substrate solution (1 mL/strip or 120  $\mu$ L/well) and place into a separate container. Add 100  $\mu$ L to each microwell. Incubate at room temperature for 10 minutes. Cover to avoid direct light.
12. Measure the required volume of stop solution (1 mL/strip or 120  $\mu$ L/well) and place in a separate container. Add 100  $\mu$ L in the same sequence and at the same pace as the substrate solution was added.
13. 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.
14. Setting the zero standard as 100% binding ( $B_0$ ), calculate % binding (%B) for each standard and sample as a percentage of the zero binding ( $\%B/B_0$ ).

## Interpretation of Results

Construct a dose-response curve using the OD values expressed as a percentage ( $\%B/B_0$ ) 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 1:5, 1:10, 1:20 or 1:100 ratio by extraction solvent as instructed in the extraction procedure and also 1:10 in wash buffer (except no dilution for infant and toddler formulas and cereal). Therefore, 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, Soybean, Chili, Cilantro and Coriander	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 - 2 ppb, 1 - 20 ppb, 2 - 40 ppb, 4 - 80 ppb, or 20 - 400 ppb depending on the dilution factors (see extraction procedure section). If a sample contains aflatoxin at a greater concentration than the highest standard, it should be diluted appropriately in extraction solvent (at the step of sample preparation) 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).

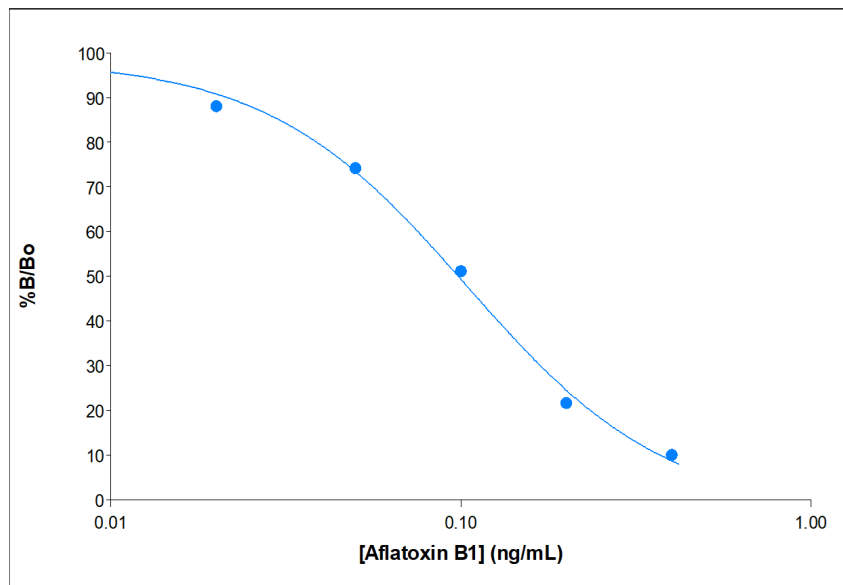


### Assay Characteristics

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

Standard (ng/mL)	%B/B <sub>0</sub>	CV (%)
0	100.0	-
0.02	88.6	2.6
0.05	74.2	3.3
0.1	51.0	5.9
0.2	21.8	11.3
0.4	9.9	5.0

The below figure is a representative standard curve for aflatoxin B1 based on the above data table.



As an example of a high matrix effect commodity, 13 silage samples, 5 corn, 2 wheat, 3 hay and 3 snaplage, which had measured less than 1 ppb for B1, B2, G1 and G2 by HPLC, were extracted with either 80% methanol or 80% acetonitrile.

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

The average recovery of a 5 ng/g (ppb) spike into four of the silage samples was as follows:

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



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

Recoveries of 5 ng/mL spiked into three soy sauce or soybean samples extracted with 80% acetonitrile were as follows based on the average of four independent experiments:

Type of Commodity	ng/mL	% Recovery
Soy Sauce	5.1	102
Soybean	4.6	92

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

Type of Commodity	ng/mL	% Recovery
Infant Milk Formula	0.49	98
Toddler Milk Formula	0.48	95
Toddler Rice Cereal	0.44	88

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

Type of Commodity	ng/mL	% Recovery
Chili Powder	4.8	95.3
Cilantro Seed	4.8	95.0
Coriander Seed	5.1	101.3

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

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

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



## Cross Reactivity

The assay will cross-react with aflatoxin analogues as follows:

- B1 - 100%
- B2 - 77%
- G1 - 64%
- G2 - 25%

## References

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- 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.

## Technical Assistance

For questions or comments, please contact your local distributor. You can also email [techsupport@hygiena.com](mailto: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>.