Helica® Zearalenone Low Matrix ELISA

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For the quantitative detection of zearalenone in cereal crops, such as maize, barley, oats, wheat, rice, and sorghum, as well as in animal feeds.

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Introduction – Zearalenone

Zearalenone (RAL/F-2 mycotoxin) is a potent, non-steroidal, estrogenic metabolite produced by several fungal genera, specifically *Gibberella* and *Fusarium*. Some *Fusarium* species are known to produce a diversity of harmful mycotoxins, including zearalenone, fumonisin, and deoxynivalenol, which result in severe health implications in domestic animals. Some more common adverse effects include breeding problems and infertility in farm animals, with female swine being the most susceptible to these estrogenic effects. Zearalenone is frequently found in cereal crops, including maize, barley, oats, wheat, rice and sorghum. Due to the wide range of commodities in which zearalenone can be detected, an analytical method such as ELISA is a useful means to provide rapid screening and identification of samples containing high levels of zearalenone.

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 and corn, designed to protect humans and animals from dangerous side effects of mycotoxins.

The Helica Zearalenone Low Matrix ELISA assay is a competitive enzyme-linked immunoassay intended for the quantitative detection of zearalenone in cereal crops, such as maize, barley, oats, wheat, rice, and sorghum, as well as in animal feeds.

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. 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 the supervision of technically qualified persons.

Principle of the Method

The Helica Zearalenone Low Matrix ELISA assay is a solid-phase competitive inhibition enzyme immunoassay. A zearalenone-specific antibody optimized to react with zearalenone is coated to a polystyrene microwell. Toxins are extracted from a ground sample with 70% methanol. If zearalenone is present it will bind to the coated antibody. After wells are decanted and washed, zearalenone bound to horseradish peroxidase (HRP) is added and binds to the antibody not already occupied by zearalenone present in the sample or standard. After this incubation period, the contents of the wells are decanted, washed and a chromogenic 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 zearalenone in the standard or sample. Therefore, as the concentration of zearalenone 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-zearalenone 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	1.5 mL/vial of zearalenone at the following concentrations: 0.0, 0.1, 0.3, 0.6, 1.2 and 4.0 ng/mL in 70% methanol, *Ready-to-Use*.
1X Bottle	Conjugate	12 mL of HRP-conjugated zearalenone in buffer with preservative, *Ready-to-Use*.
2X Bottles	Assay diluent	2 x 12 mL proprietary sample diluent, *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% Tween20®*, bring to 1 Liter with distilled water and store refrigerated. (Wash buffer)

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Materials Required but Not Provided

- Grinder sufficient to render sample to a particle size of fine instant coffee
- Sealable extraction container: Minimum 125 mL capacity
- Balance: up to 20 g measuring capability
- Graduated cylinder: 100 mL
- Methanol: 70 mL reagent grade per sample
- Distilled or deionized water: 30 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 (substrate) are photosensitive and are packaged in light-protective opaque bottles. Store bottles in the dark, avoid exposure to direct light 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 return unused reagents to their original bottles. The assay procedure details the 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.

- Read this manual carefully before starting the test. The assay must be performed by specialized and trained staff.
- 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 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

Cereals

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 (70% methanol) by adding 30 mL of distilled or deionized water to 70 mL of methanol (reagent grade) for each sample to be tested.
3. Weigh out a 20 g ground portion of the sample into a sealable extraction container or blender and add 100 mL of the Extraction Solvent (70%). *Note: The ratio of sample to extraction solvent is 1:5 (w/v).*
4. Mix by shaking or blending in a sealed container or a blender for a minimum of 3 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 70% methanol.
7. The sample is now ready for testing.
8. The final dilution for use in calculations is **1:50**.

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 (70% methanol) by adding 30 mL of distilled or deionized water to 70 mL of methanol for each sample to be tested.
3. Transfer 100 mL of 70% methanol to a sealable 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 the sealed container for a minimum of 3 minutes.
5. Allow the particulate matter to settle, then filter 5-10 mL of the extract through a Whatman #1 filter and collect the filtrate to be tested.
6. Dilute an aliquot of the extract 1:50 with 70% methanol.
7. The sample is now ready for testing.
8. The final dilution for use in calculations is **1:250**.

Assay Procedure

Note: It is recommended that a multi-channel pipettor be used to perform the assay. If a single-channel pipettor is used, it is recommended that no more than a total of 16 samples and standards (2 test strips) are run.

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 twice the number of antibody-coated microtiter wells in another microwell holder to run duplicates. 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. Dispense 200 µL of the Assay Diluent into each mixing well.
5. Using a new pipette tip for each, add 100 µL of each standard and prepared sample to the appropriate mixing well containing diluent. Mix by priming pipettor at least 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 µL of contents from each mixing well to a corresponding antibody-coated microtiter well. Incubate at room temperature for 10 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 3 washes.
8. Tap the microwells (face down) on a layer of absorbent towels to remove residual wash buffer.
9. Measure the required volume of Zearalenone-HRP conjugate (1 mL/strip or 120 µL/well) and place in a separate container. Add 100 µL of the conjugate to each antibody-coated well and incubate at room temperature for 10 minutes. Cover to avoid direct light.
10. Repeat steps 7 and 8.
11. Measure the required volume of substrate reagent (1 mL/strip or 120 µL/well) and place into a separate container. Add 100 µL 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 µL/well) and place into a separate container. Add 100 µL in the same sequence and at the same pace as the substrate reagent was added.
13. Read the optical density (OD) of each microwell with a microtiter plate reader using a 450 nm filter. Record the optical density (OD) of each microwell.
14. Setting the zero standard as 100% binding (B_o), calculate % binding (%B) for each standard and sample as a percentage of the zero binding (%B/B_o).

Interpretation of Results

Construct a dose-response curve using the OD values expressed as a percentage (%B/B_o) of the OD of the zero (0.0 ng/mL) standard against the zearalenone 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 ratio with 70% methanol in the extraction procedure, followed by an additional dilution of 1:10 or 1:50 in 70% methanol. Therefore, the level of zearalenone shown by the standard must be multiplied by 50 or 250, respectively, in order to indicate the ng per gram (ppb) of commodity as follows.

Standard (ng/mL)	Cereal diluted 1:50 (ppb in sample)	Animal feed diluted 1:250 (ppb in sample)
0.0	0.0	0.0
0.1	5.0	25.0
0.3	15.0	75.0
0.6	30.0	150.0
1.2	60.0	300.0
4.0	200.0	1000.0

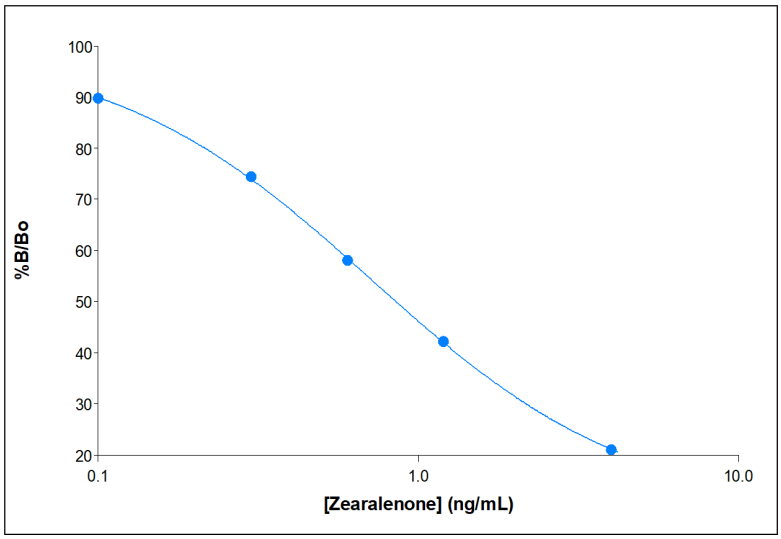
If a sample contains zearalenone at a greater concentration than the highest standard, it should be diluted appropriately in extraction solvent and retested. The extra dilution step should be taken into consideration when expressing the final result.

Assay Characteristics

Data from twelve consecutive standard curves gave the following results:

Standard (ng/mL)	%B/B$_0$	CV (%)
0.0	100.0	-
0.1	89.4	2.4
0.3	74.6	3.8
0.6	57.9	4.5
1.2	42.9	6.3
4.0	29.2	7.7

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



Recoveries of 500 ppb, 150 ppb and 60 ppb zearalenone spiked into feed samples were as follows based on an average of six independent experiments (n=6).

Feed sample spike (ppb)	% Recovery
500	81.5
150	97.9
60	117.8

Recoveries from certified reference material (corn) were as follows based on an average of six independent experiments (n=6).

Corn (Reference material) (ppb)	% Recovery
273	88.8
121	102.1

Cross-reactivity

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

Compound	% Cross-reactivity
Zearalenone	100
α-Zearalanol	6
α-Zearalenol	7
β-Zearalanol	9
β-Zearalenol	80
Zearalanone	5

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.