# ZEARALENONE ELISA ASSAY – LOW MATRIX

**(Cat. No. 981ZEA01LM-96)**

## INTRODUCTION

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 of the 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 zearalenone can be detected in, analytical methods such as ELISA is a useful means to provide rapid screening and identification of samples containing high levels of zearalenone.

## INTENDED USE

The HELICA Zearalenone 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.

## 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 Zearalenone 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 horse-radish 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 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 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 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

| | | | |
|---|---|---|---|
| 1 X 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.* |
| 1 X Plate | **Mixing Wells** | Green | 96 non-coated wells (12 eight well strips) in a microwell holder, *Ready-to-Use.* |
| 6 X Vial | **Zearalenone Standards** | Black Cap | 1.5mL/vial of zearalenone at the following concentrations: 0.0, 0.1, 0.3, 0.6, 1.2, 4.0ng/mL in 70% methanol, *Ready-to-Use*. |
| 1 X Bottle | **Zearalenone HRP-conjugate** | Green Cap | 12mL of zearalenone conjugated to peroxidase in buffer with preservative, *Ready-to-Use*. |
| 2 X Bottle | **Assay Diluent** | Brown Cap | 2 × 12mL proprietary sample diluent, *Ready-to-Use*. |
| 1 X Bottle | **Substrate Reagent** | Blue Cap | 12mL stabilized tetramethylbenzidine (TMB), *Ready-to-Use*. |
| 1 X Bottle | **Stop Solution** | Red Cap | 12mL Acidic Solution, *Ready-to-Use*. |
| 1 X Pouch | **Washing buffer** | | PBS with 0.05% Tween 20, bring 1 liter with distilled water and store refrigerated. |

## MATERIALS REQUIRED BUT NOT PROVIDED

### Extraction Procedure

- Grinder sufficient to render sample to particle size of fine instant coffee
- Collection Container: Minimum 125mL capacity
- Balance: 20g measuring capability
- Graduated cylinder: 100mL
- Methanol: 70mL reagent grade per sample
- Distilled or deionized water: 30mL 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 (19° - 27°C) 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 zearalenone. 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 1/10$^{th}$ 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 technique.**

Cereals

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 30mL of distilled or deionized water to 70mL of methanol 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 3 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 70% methanol.
7. The sample is now ready. The standards require no pre-dilution before use.
8. Final dilution for use in calculation = 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 30mL of distilled or deionized water to 70mL of methanol 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 3 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 1:50 with 70% methanol.
7. The sample is now ready. The standards require no pre-dilution before use.
8. Final dilution for use in calculation = 1:250

## ASSAY PROCEDURE

**Note: It is recommended that a multi-channel pipettor be utilized 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. Reconstitute the PBS-Tween packet by washing out the contents with a gentle stream of distilled or deionized water into a 1-Liter container. Q.S. 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 microwells in another microwell holder to run standards and samples in duplicates.
3. Dispense 200µL of the Sample Diluent into each mixing well.
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 10 minutes. The mixing wells contain enough solution to run each standard and/or sample in duplicate. If running each standard and/or sample in singlets or more replicates is desired, 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-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. Measure the required volume of Zearalenone HRP-conjugate (1mL/strip or 120µL/well) and place in a separate container. Add 100µL of conjugate to each microwell. Incubate at room temperature for 10 minutes.
9. Repeat steps 6 and 7.
10. Measure the required volume of Substrate Reagent (1mL/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. Measured the required volume of Stop Solution (1mL/strip or 120µL/well) and place in a separate container. Add 100µL in the same sequence and at the same pace as the Substrate Reagent 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 the % binding (%B) for each standard and sample as a percentage of the zero binding (%B/Bo).

## INTERPRETATION OF RESULTS

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 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 5:1 ratio with 70% methanol in the EXTRACTION PROCEDURE followed by an additional dilution of 10:1 or 50:1 in 70% methanol, and so 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 |

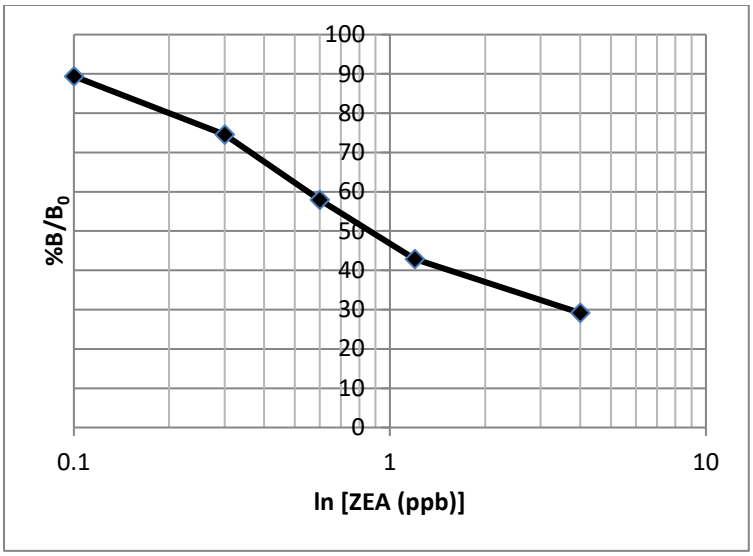
If a sample contains zearalenone at greater concentration than the highest standard, it should be diluted appropriately in extraction solvent and retested. The extra dilution step should be taken into consideration when expressing the final result.

## 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 500ppb, 150ppb, and 60ppb zearalenone spiked into feed samples were as follows based on six independent experiments (n=6):

| ppb | % Recovery |
|---|---|
| 500 | 81.5 |
| 150 | 97.9 |
| 60 | 117.8 |

Recoveries from certified reference material (corn) were as follows based on six independent experiments (n=6):

| ppb | % Recovery |
|---|---|
| 273 | 88.8 |
| 121 | 102.1 |

**CROSS-REACTIONS**

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 |