ZEARALENONE TESTING

1. PURPOSE

This directive provides information concerning the testing for zearalenone as an official criteria factor under the United States Grain Standards Act (USGSA), as amended.

2. REPLACEMENT HIGHLIGHTS

This directive is revised to include additional approved commodities to the grain/commodity list for the Charm Sciences Rosa® test kit. The commodities include Barley, Flaking Corn Grits, Oats, Rough Rice, and Wheat Flour. This directive supersedes FGIS Directive 9180.66, "Zearalenone Testing", dated, June 15, 2007.

3. BACKGROUND

Zearalenone is the generic name for a mycotoxin that is produced by several species of mold (*Fusarium),* the most notable of which is the *Fusarium graminearum.* Species of Fusarium mold are common and widespread in nature, and are found worldwide on most economic crops. Fusarium mold infection and production of zearalenone are most notable on corn, wheat, sorghum, oats, and barley.

Historically, zearalenone has been found at low levels in corn, barley, oats, sorghum, corn meal, hay, silage, and commercial animal rations in the United States. A combination of the fungus strain, high moisture content, and a period of relatively high temperature (as in ears of corn before harvest) followed by a period of low temperature (as in corn stored on the ear in cribs) favor the production of the toxin.

There are no zearalenone action limits, guidance, or advisory levels established by the Food and Drug Administration (FDA) at this time. Official personnel must adhere to approved test kit conformance limits, and service thresholds (e.g., 50 ppb) requested by applicant.

4. TESTING SERVICE

All official zearalenone testing is performed as prescribed in this directive by authorized employees of the Federal Grain Inspection Service (FGIS) or official service providers. Testing performed on standardized grains (e.g., corn, wheat, barley, and oats) is performed as an official criteria factor under the authority of the United States Grain Standards Act (USGSA), as amended. Testing performed on processed grain products (e.g., flaking corn grits, milled rice,) and other commodities is provided under the authority of the Agricultural Marketing Act (AMA) of 1946, as amended.

Individuals who wish official zearalenone testing should contact the nearest FGIS field office or official service providers.

Detection and quantification limits are between 50 - 1,000 parts per billion (ppb).

5. TYPES OF SERVICES

Three types of zearalenone testing services are available as follows:

a. Submitted Sample Service - Analysis based on a sample submitted by the applicant for service.

b. Official Sample-Lot Service - Analysis based on an official sample obtained by official personnel and analyzed by official personnel.

(1) Single Lot Inspection.

Samples may be obtained and tested on either an individual carrier basis or a composite sample basis (maximum of five railcars or fifteen trucks per composite sample).

(2) Unit Train Inspection under the CuSum Loading Plan.

Unit trains are analyzed on a sublot basis for corn. Acceptable sublots must conform to contract specifications when "maximum" limits are specified.

For unit trains, the sublot size for zearalenone testing and for grade analysis may be different. For example, an applicant may request grade analysis on the basis of a sublot containing two cars and request zearalenone analysis on the basis of five cars.

The maximum size sublot for zearalenone testing is 5 railcars for unit trains consisting of less than 200,000 bushels, or less than 50 cars. For unit trains consisting of 200,000 bushels or more, or 50 railcars or more, the maximum sublot size is 10 railcars.

(3) Export Shiplots.

Export shiplots are analyzed on a sublot basis for corn. Acceptable sublots must conform to contract specifications when "maximum" limits are specified.

The testing frequency for shiplot grain will be the same as the sample for grade analysis unless the applicant specifically requests zearalenone analysis on the basis of a component sample.

(4) Supplemental Testing.

Upon request, supplemental testing may be performed as follows:

Composite samples may be analyzed in addition to the sublot test for corn shiplots or unit trains.

(5) Alternate Testing.

Upon request, alternate testing methods may be used, provided that the minimum testing requirements are met. Examples of alternate testing are as follows:

Grain shipments may be tested on a component sample basis in lieu of the sublot basis under the provisions of Book III, Inspection Procedures. Components are combined and averaged to determine the sublot result. Component samples will not be designated as material portions due to zearalenone because the Food and Drug Administration (FDA) has not established action limits at this time. Acceptable quality will be based on the sublot result as compared to the contracted "maximum" specification.

c. Warehouseman Sample-Lot Inspection Service - Analysis based on an official sample obtained by a licensed warehouseman sampler and analyzed by official agency/field office, as applicable.

6. REVIEW INSPECTIONS

7CFR Part 800.125 and 800.135 of the USGSA permit a review (reinspection, appeal, Board appeal) inspection on either official grade/factors or official criteria. When requested, a review inspection for official grade or official factor and official criteria may be handled separately even though both sets of results are reported on the same certificate.

Review inspection services for zearalenone are provided on either a new sample or the file sample in accordance with the regulations. Board appeal inspection services are limited to the analysis of file samples.

7. APPROVED TESTING METHOD

The methods listed below have been conformance tested to perform within FGIS specifications. All test methods has been certified to provide quantitative Zearalenone analysis that are accurate up to the conformance test level at which it was approved.

Any test results that are above the established conformance limits are reported as exceeding the conformance limit.

FGIS Approved Test Methods		
Method and Test Kit	Test Format	Conformance Limit
Charm Sciences Rosa® zearalenone (Quantitative)	Lateral Flow Strip	50 - 1000 PPB
Ridascreen® Fast zearalenone SC (Quantitative)	Competitive enzyme immunoassay (ELISA)	50 - 1000 PPB

Kits Approved for Quantitative Analysis.

Listed in the table below are the test kits that are commonly used for official quantitative zearalenone analysis. Use the table to determine the appropriate test kit(s) to use for testing the listed grain/commodity. For information the testing of mixed grain, contact the Policies and Procedures ranch.

GRAIN/COMMODITY	TEST METHOD	
	Charm Rosa® Quantitative	Ridascreen Fast zearalenone SC
Corn	X	X
Sorghum	X	
Wheat	X	
Wheat Flour	X	
Barley	X	
Milled Rice	X	
Rough Rice	X	
Cracked Corn	X	X
Distiller Dried Grains w/Solubles	X	
Flaking Corn Grits	X	
Oats	X	

NOTE: An X entered into a block denotes that the test kit has been evaluated and approved for the grain/commodity.

8. DISCLAIMER CLAUSE

The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

9. WORK AREA REQUIREMENTS

The work area requirements covered under this section apply to FGIS-occupied space only.

a. Sample Grinding Area.

Samples must be ground in space separate from the analytical space. The field office manager and safety officer must determine whether added ventilation or a dust removal device is needed in the grinding area to remove airborne dust particles. Refer to the GIPSA Safety and Health Office in Washington, D.C., for assistance in determining whether added dust removal equipment (e.g., exhaust fan) is required.

b. Sample Testing Area.

Test methods that involve the use of volatile chemicals (e.g., methanol) must be performed in FGIS-approved laboratory space.

10. FGIS LABORATORY REQUIREMENTS

FGIS-approved laboratories are required for mycotoxin testing that involves the use of hazardous materials (e.g., flammable liquids). The requirements covered under this section apply to FGIS-occupied space that is dedicated for the sole function of mycotoxin testing.

Zearalenone testing methods require the use of flammable liquids and suspected carcinogens. The building owner must permit the use of chemicals (e.g., acetonitrile, methanol) in space used by FGIS. FGIS will provide testing services onsite only in facilities that provide protection to FGIS personnel.

Individual elevators may provide two kinds of space for FGIS personnel to perform onsite zearalenone testing. The space may be located (1) in a building along with other occupants, or (2) in a building devoted exclusively to laboratory space.

In either case, the plan for the intended laboratory space is subject to inspection and approval by FGIS prior to construction. The Safety and Health Office and field office manager will review proposed plans and suggest ways to comply with the requirements.

The following are minimum requirements for FGIS-occupied laboratory space.

a. Location.

Locate the laboratory at least 100 feet from the base of the elevator headhouse. This distance is subject to negotiation when the elevator uses exterior grain legs and/or inclined belts in lieu of interior grain legs or where the headhouse is equipped with blow-out panels or the headhouse consists of a lightly covered framework.

Laboratories must meet the following requirements when they are located in a building with other occupants:

(1) Isolate the laboratory from non-laboratory occupants using a fire barrier having at least a 1-hour fire resistance.

(2) Provide a fire barrier consisting of floors, ceilings, and interior walls.

(3) Provide all passageways and other openings that lead to adjacent interior space with self-closing fire doors having a 1-hour fire resistance. Do not block these doors open.

(4) Separate the space from central heating, ventilation, and air-conditioning using automatic-closing fire dampers in the heating, ventilation, and air-conditioning ducts near the fire-barrier, or provide a separate heating, ventilation, and air-conditioning system in the laboratory.

b. Size.

Dedicate the space strictly for laboratory (chemical) work. Supply adequate space for chemical analysis (minimum of 100 square feet).

c. Electrical System.

Provide the laboratory space with electrical power and lighting meeting the standards of the National Electrical Code. Wiring suitable for Class I location is not required. A three-wire system consisting of an energized wire, a neutral wire, and a grounding conductor is satisfactory. Install overhead lighting fixtures through ceilings that serve as fire barriers. Fixtures suspended below such ceilings are acceptable.

d. Plumbing.

Provide the laboratory space with a basin having hot and cold portable water and a sewer connection.

e. Exhaust System.

The exhaust system must remove chemical vapors from the work area. Normal air conditioning and heating may provide adequate ventilation when performing testing procedures in a building devoted exclusively for laboratory space. Refer to the GIPSA Safety and Health Office in Washington, D.C., for assistance in determining whether added ventilation, such as a fume hood, is needed. If needed, situate the laboratory space so that hoods are vented to the exterior of the building. Fume hood ventilation will require a 6 or 8-inch diameter opening, either vertically through the ceiling and roof or horizontally through an exterior wall. In some cases, a portable hood may be sufficient.

f. Eyewash and Safety Shower Station.

Provide the laboratory space with eyewash equipment (eyewash bottle or permanent faucet-mounted fixture). A permanent, faucet-mounted eyewash fixture is highly recommended.

g. Cautionary Markings.

Provide signs for the laboratory door(s) as follows:

(1) "Biohazardous Material Present"

(2) "No Smoking, Eating, or Drinking"

(3) "Flammable Material Present"

(4) "Wear Safety Protection"

(5) "Admittance of Authorized Personnel Only"

(6) Refrigerator Signs

Provide signs for the refrigerator used for storing test kits, chemicals, or solutions, as follows:

(a) "Biohazardous Material Present"

(b) "No Food or Drink to be Stored in this Refrigerator"

For further information concerning the laboratory space requirements, contact the FGIS Safety and Health Office.

11. SAFETY

FGIS employees must comply with good practices to ensure a safe and efficient work environment. To accomplish this, include the following as part of an overall FGIS laboratory/testing area "Standard Operating Procedure" (SOP). Maintain the SOP, this handbook, and current Material Safety Data Sheets (MSDS) at each laboratory/testing location.

During onsite supervision at agency locations, FGIS employees must assess their personal safety requirements. If personal safety is questionable, FGIS employees must determine if personal protective equipment can be used to correct the safety deficiency at the testing location. If FGIS employees cannot utilize personal protective equipment to provide for a safe work environment, then onsite zearalenone supervision must occur only when the testing area is considered safe. Interested persons are restricted from entering the zearalenone testing area during testing unless accompanied by official personnel and must observe the health and safety rules while in the area.

FGIS personnel must abide by the following safety practices when performing testing in an FGIS-approved laboratory.

a. Do not smoke, eat, drink, chew gum or tobacco in the laboratory.

b. Wash hands immediately before and after eating, drinking, and smoking.

c. Wear the following protective equipment: disposable, fire-retardant laboratory coat; disposable, impermeable gloves; safety glasses or splash goggles.

d. Wear an FGIS-approved disposable mask and hair protection when exposed to airborne grain dust.

e. Do not store food or drink in the laboratory refrigerator used for storing chemicals, solutions, and test kits.

f. Do not store masks and hair protectors in the grinding area where they might become contaminated by the dust particles.

g. Label all bottles and containers according to the Hazard Communication Program and the Chemical Hygiene Plan. In addition, when preparing mixtures of solutions, securely apply a label with the name of the solution, the preparation date, and the preparer's initials written in permanent ink.

h. Store equipment outside the fume hood in a manner that will not clutter bench tops or obstruct movement.

i. Prepare all chemical solutions and perform chemical analyses under a working fume hood.

j. Limit the total quantity of waste chemicals in the laboratory to one liquid gallon.

k. Limit the total amount of flammable solvent (including waste) in the laboratory to two gallons.

l. Maintain a current MSDS for each chemical in the laboratory. If each supply of chemicals received does not have an MSDS enclosed, contact the company and request one immediately.

m. Store flammable solvents in an approved storage cabinet.

n. Store waste chemicals (e.g., methanol) in impermeable metal containers meeting Underwriters Laboratory approval for Class I liquids. The containers must be capable of maintaining a tight seal and must be labeled "Flammable" or "Biohazardous Material" or both, as applicable.

o. Contact an Environmental Protection Agency (EPA)-approved or EPA-certified waste disposal company and make arrangements for removal of chemical wastes or provide other suitable waste disposal procedures consistent with existing laws that do not create a hazard to the community.

12. SANITATION REQUIREMENTS

The sanitation requirements for spillage, labware, and excess sample extract listed in this section are applicable to testing performed at an FGIS-approved laboratory. Official agencies must adhere to the requirements for cleaning labware and should follow procedures established in their area for the disposal of excess sample extract.

Perform the following procedures only while wearing disposable, impermeable gloves, chemical splash goggles, and a fire-retardant laboratory coat. If hands become contaminated, wash immediately with soap and water.

a. Spillage.

Clean areas and materials contaminated by any extraction solution spills. Wipe up the affected areas using an absorbent cloth or paper towels, then wash the area with a soap/water solution. Place cleaning materials in a plastic waste bag, close tightly, and discard in a dumpster or landfill disposal site.

b. Labware.

Prepare a solution consisting of dishwashing liquid and water. Completely submerge the used glassware, funnels, beakers, etc., wash thoroughly, then rinse with clean water before reusing.

c. Excess Sample Extract.

All sample extracts containing chemicals such as methanol should be treated as hazardous chemicals and disposed of in the chemical waste container. Refer to the appropriate testing procedures for specific waste disposal instructions.

13. SAMPLE SIZE

The manner in which samples are obtained and processed is an important consideration when testing for mycotoxins. To ensure that the test results accurately reflect the zearalenone concentration present in a lot, samples must be representative of the lot and of sufficient size to compensate for uneven distribution of the contaminant. Obtain samples according to the guidelines in the Grain Inspection Handbook, Book I, "Grain Sampling."

The minimum sample size is based on the type of lot. Applicants may request a sample size larger than the minimum sample size.

Lot Type	Minimum Sample Size
Trucks	2 pounds (approximately 908-grams)
Railcars	3 pounds (approximately 1,362-grams)
Barges/Sublots	10 pounds (approximately 4,540-grams)
Submitted Samples	10 pounds / approximately 4,540-grams **(recommended)**

NOTE: A minimum sample size of 10 pounds is required for composite type samples (e.g., a single sample representing multiple carriers). A 10-pound sample size is also recommended, but not required, for submitted samples.

Testing locations that receive submitted samples that contain less than the recommended 10-pound sample size must grind the entire sample as submitted. For submitted samples that are 10-pounds or more, a minimum of 10-pounds must be ground for testing purposes.

14. SAMPLE PREPARATION

a. Subportions.

Grind the entire sample obtained for zearalenone testing and prepare two 500-gram subportions from the ground sample. Prepare a 500-gram work portion for original testing services and a 500-gram file sample portion for review testing. For submitted samples, retain as large a sample as possible.

From the 500-gram work portion, obtain a 50-gram test portion and weigh on an FGIS-approved type scale with a minimum division size of 0.1-gram, using one of the following options.

(1) Collect the 500-gram sample and divide (using an approved divider) out a 50-gram test portion for analysis. Maintain the balance as a file sample.

Or

(2) Collect the 500-gram sample in a clean container and stir/mix the sample with a spatula or spoon for about 30 seconds ensuring a homogeneous blend (low to high). Using a spatula or spoon dip out a 50-gram test portion for analysis. Maintain the balance as a file sample.

b. Saving File Samples.

Maintain file samples for all lots/samples that do not meet the contractual specification of the applicant for service, or as required for the local area monitoring program.

When applicable, maintain a representative file sample for each lot, sublot, composite, or submitted sample tested. For submitted samples that are less than 500-grams, retain as large a sample as possible. For information concerning file sample retention periods refer to FGIS Directive 9170.13, "Uniform File Sample Retention System".

c. Storing File Samples.

If file samples are required, store each sample in a manner that will maintain the integrity of the sample and prevent manipulation or substitution. Place the sample in paper bags or envelopes and label each file sample with the test date and identification. Take precautions to ensure that file sample containers are strong enough to prevent loss of sample integrity when storing samples. Do not store samples near heat, windows, or in direct sunlight. (Store samples in cold storage if available.)

15. OPERATION OF GRINDERS

Samples must be ground to a fine particle size that is sufficiently fine enough to obtain a homogeneous blend. Avoid over-grinding or pulverizing a sample because it produces an excessively powdery mix that will slow down the filtration process.

Grinding must be performed in an area separate from the testing area. Use the Romer Mill - Model 2A, Bunn Grinder, or equivalent to grind the sample.

FGIS employees must follow the manufacturer's safety procedures for operating the grinder and must wear protective equipment (i.e., lab coat, mask, gloves, and hairnet) when grinding samples.

Zearlenone samples that contain an excessive amount of moisture content (above 20%) are problematic to the zearalenone grinding and testing procedures. High moisture corn does not grind to a suitable particle size therefore affecting the accuracy of the test results. Therefore, official personnel must ensure that high moisture corn samples are allowed to **naturally dry** to a moisture level of **20% or less before grinding and testing.**

a. Romer Mill.

(1) General Operating Instructions.

The Romer Mill simultaneously grinds and sub-samples corn at the rate of approximately 1 pound per minute. An adjustable restrictor door located above the collection chute varies the amount of ground sample allowed into the collection chute. Official personnel must adjust the grinder to obtain the required testing and file portions from the sample.

Note: DO NOT dip out the 500-gram portion used for work and file samples.

Adjust the grinder by locating the first line (far left) etched on the restrictor door. Position the door approximately 1/3 of the way between the first and second line. For a 10-pound sample, approximately 500-grams will be collected through the collection chute.

Once the grinder is adjusted to obtain the 500-gram sample, mark the location of the setting. To increase the sample size, move the restrictor door to the left.

If a composite sample is required in addition to sublot-by-sublot analysis, adjust portion sizes as needed to obtain an adequate size composite and still maintain individual file samples. Obtain the composite sample from the ground sublot samples.

(2) Grinding the Sample.

Grind the entire 10-pound sample with the grind lever set at the finest range. If the grinder is experiencing difficulty (e.g., over-heating, bogging down) at the fine setting, change the grind lever to the coarsest setting. After grinding the remainder of the sample at the coarsest setting, switch the setting back to fine. Collect the entire 10-pound portion and regrind at the fine setting.

Note: If the grinder motor overheats the breaker switch will release and turn off the power to the grinder. If this occurs, allow the motor to cool and then set the grind lever the coarsest setting. Do not grind samples with moisture content of 20 percent or more.

b. Bunn Grinder.

(1) General Operating Instructions.

The Bunn-O-Matic grinds corn at a rate of approximately 2 pounds per minute and has a holding capacity of approximately 3 to 4 pounds when fully closed. Official personnel must grind the entire sample and cut it down (using an FGIS-approved divider) to obtain the required testing and file portions from the sample.

Note: DO NOT dip out the 500-gram portion used for work and file samples.

(2) Grinding Samples.

Grind the entire 10-pound sample with the grind lever set at the fine selection. Add 3 to 4 pounds at a time into the hopper until all 10 pounds are ground. If the grinder is experiencing difficulty (e.g., over-heating, bogging down) at the fine setting, change the grind lever to the coarse setting. After grinding the remainder of the sample at the coarse setting, switch the setting back to fine. Collect the entire 10-pound portion and regrind at the fine setting.

Note: If the grinder motor overheats the breaker switch will release and turn off the power to the grinder. If this occurs, allow the motor to cool and then set the grind lever to the coarse setting. Do not grind samples with moisture content of 20 percent or more.

Obtain the composite sample from the ground sublot samples. Official personnel must use an approved divider to reduce the size of the ground portion to the stated 500-gram work and file samples.

c. Cleaning Grinders.

A small amount of ground sample will remain in the grinder after the total sample has been ground. To prevent the contamination of subsequent samples, clean the grinder using one of the following cleaning procedures:

(1) If a Vacuum Cleaner is Available.

After a sample has been ground and collected, with the unit turned on, use a vacuum cleaner with an attachment that will fit over the mouth of the chute(s). Place the attachment at the bottom of each chute for about 30 seconds. After cleaning the chute(s), turn the power off and prepare for the next sample.

(2) If a Vacuum Cleaner is not Available.

Clear the grinder by discarding a small portion (first 10 - 15 grams) of the next sample to be tested.

(a) Pour the sample into the grinder and turn it on long enough to collect the first 10 - 15-grams.

(b) Turn the power off, and discard the 10 - 15-grams ground sample.

(c) Turn the power back on and finish grinding the sample to collect the remaining sub-sample for analysis.

16. CHECKING PARTICLE SIZE

a. Procedures for Checking the Performance of the Grinder.

For locations that perform mycotoxin testing on coarse (e.g., corn) and small grains, perform the check with a 100-gram sample portion of corn using the following procedures.

(1) Grind a sample portion of approximately 100-grams of corn having a moisture content of 14.0 percent or less.

(2) Weigh the entire portion that was ground.

(3) Sieve the portion across a standard No. 20 wire woven sieve.

(4) Weigh the portion that passed through the sieve.

(5) Determine the percent of fine material, by weight, as follows:

Fines = weight from step (4) divided by the weight from step (2) X 100.

Locations performing mycotoxin testing on small grains only, perform the particle size check using a 100-gram sample portion of wheat (dockage-free) having a moisture content of 13 percent or less.

b. Optimum Particle Size.

The optimum range for particles of coarse and small grain passing through the No. 20 sieve is between 60 and 75 percent. Whenever the ground particles appear to be too coarse, or the results of a grinder check indicate that less than 50 percent of the ground portion passes through the No. 20 sieve, the grinder should be adjusted or repaired to meet the optimum range requirements.

Grinding apparatuses must be checked periodically to determine whether they are producing a final product that meets the particle size requirements as listed above. Official personnel shall determine the frequency of the checks based on a number of items that include visual observation of the ground product, number of samples ground since last check, and time (number of days) since the last check was performed. Record all particle check results in a convenient location for future reference purposes.

17. CHARM SCIENCES ROSA® ZEARALENONE (QUANTITATIVE) TEST KIT

The extraction solution and other materials used in the Charm Sciences Rosa® Zearalenone (Quantitative) test kit necessitate the use of separate FGIS-approved laboratory space. FGIS employees must comply with all applicable safety and sanitation requirements as listed in this directive to ensure a safe and efficient work environment.

a. Preparation of Extraction Solution.

The extraction solvent used in the ROSA® zearalenone (Quantitative) test method is a methanol/water (distilled or deionized) mixture consisting of 70 percent methanol (Reagent grade or better) and 30 percent water.

(1) Using a graduated cylinder, measure 700 ml of methanol and place it into a clean carboy with spigot.

(2) Add 300 ml deionized or distilled water to the methanol and shake vigorously until it is completely mixed.

(3) Label the container stating the mixture (70 percent methanol and 30 percent water), date of preparation, and initials of technician who prepared the solution.

(4) Store this solution at room temperature in a tightly closed container until needed.

NOTE: To prepare smaller or larger amounts of solution use the ratio of 7 parts methanol to 3 parts of deionized or distilled water.

b. Preparation of Testing Materials.

NOTE: A Negative and Positive Control should be run periodically to verify performance of equipment and test strips (daily, weekly, bi-weekly, or monthly, based on internal quality assurance standards).

(1) Negative Control.

Add 100 µl of 70 percent methanol solution to 1.0 ml of ZEAR Dilution Buffer to prepare Negative Control Diluted Extract. Use 300 µl of the prepared extract as your sample, and test following Sample Analysis Procedures section (e). **Negative Control must read less than 15 ppb.**

(2) Positive Control.

Reconstitute/prepare the Positive Control by adding 3.0 ml of ZEAR Dilution Buffer followed by 300 µl of 70 percent methanol to the zearalenone Positive Control. Mix thoroughly and allow to stand for 10 minutes before use. Mix again before use. Use 300 µl as your diluted extract and test following Sample Analysis Procedures section (e). **Positive Control must read between 150 - 350 ppb.**

NOTE: Store at 32 - 45 °F for up to one week, or freeze at -4 °F for 2 months.

(3) Equipment Preparation.

(a) Incubator must be at 45±1°C (temperature indicator is green).

(b) Incubator must be clean and level.

(4) ZEAR Dilution Buffer.

Predispense 1.0 ml of ZEAR Dilution Buffer into a micro-centrifuge tube for each sample to be tested. Allow to reach room temperature before use and store unused portions at 32 - 45 °F.

(5) Test Strips.

(a) Remove ROSA® moisture resistant container from the refrigerator and allow it to reach room temperature to limit condensation.

(b) Remove only the number of strips to be used and return container to 32 - 45 °F storage. Strips are stable at room temperature for at least 12 hours.

NOTE: If blue desiccant packets turn white or pink, test the strips with Negative and Positive Controls before continued use.

c. Extraction Procedures.

(1) Transfer 50-grams of ground sample into a clean extraction container.

(2) Add 100 ml of the (70/30) methanol/water extraction solvent.

(3) Blend for 2 minutes. Allow sample to settle for 1 minute to obtain a clear sample extract.

NOTE: If particles are present after settling, filter or centrifuge to clarify sample extract. **To Filter:** funnel the extract through Whatman 2V (or equivalent) filter paper into a clean/labeled collection container. **To Centrifuge:** transfer 1.0-1.5 ml of sample extract to a labeled micro-centrifuge tube and centrifuge for 10 seconds. The clarified extract is now ready for testing.

d. Test Procedures.

(1) Sample Preparation.

(a) Pipette 100 µl of filtered clarified sample extract to a predispensed (1.0 ml ZEAR Dilution Buffer), labeled micro-centrifuge tube, cap, and mix. Repeat for additional samples.

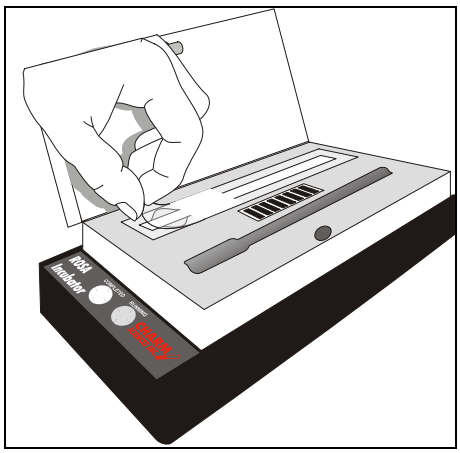
NOTE: This step must be completed within 20 minutes after extracting the sample (previous step c) or the sample extraction should be repeated.

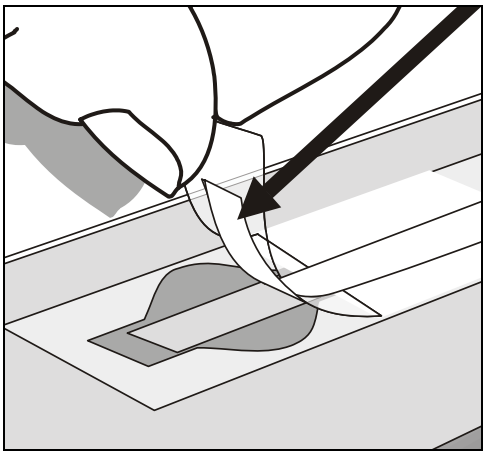
(b) Label the test strip to identify sample.

(c) Filter each sample by drawing 1 ml of the prepared sample (100 µl filtered clarified sample extract and 1.0 ml ZEAR Dilution Buffer) into a 1 ml syringe and pass the sample through a Minisart RC15 syringe filter. This is the Diluted Extract.

(2) Open the incubator lid and place test strip in the ROSA-M Incubator with the lat side facing upward.

(3) While holding the strip flat on the incubator, use tab to peel tape back to the indicated line exposing the sample pad. Avoid bending back the white wick and sponge under the tape.

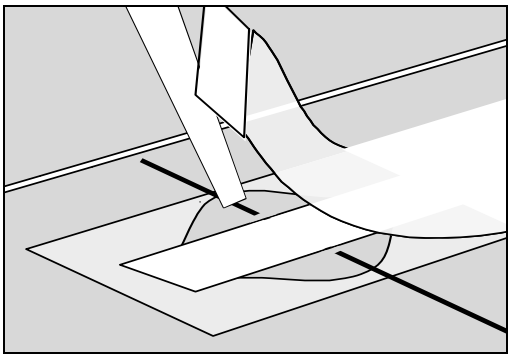




e. Sample Analysis Procedures.

(1) Pipette 300 µl of diluted extract into the side of the strip sample compartment at the position indicated by the black line on the incubator.

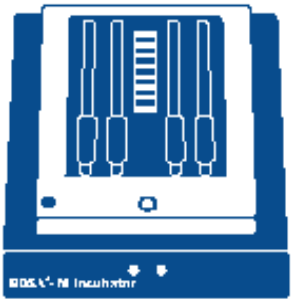
NOTE: Pipette very slowly.



(2) Reseal the tape over the sample pad compartment. When testing multiple samples, complete the peel, pipette, and reseal steps on each strip before going to the next strip.

NOTE: Add diluted extract to all strips within 1 minute. If a quad incubator is used, 4 samples can be incubated simultaneously.

(3) Close lid on the incubator and tighten the latch. The solid red timer light will automatically start when the lid is closed.



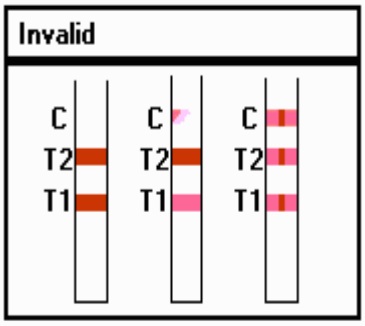
LF-INC4-45D: Quad incubator, 10-minute timer with display, set for 45° C for Test Strips

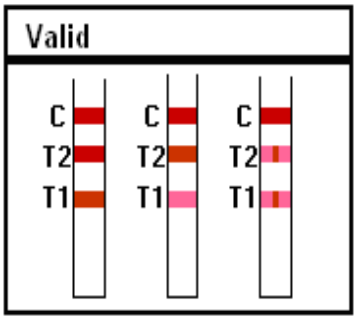
(4) Incubate for 10 minutes. After the incubation step is complete, a beeper will sound for 2 minutes, and the yellow "test complete" light will begin to flash.

(5) Remove strip(s) and interpret the results. **Strips must be removed from the incubator and read within 2 minutes of incubation completion.** After strip removal, lower but do not latch the incubator lid.

f. Visually Interpreting the Lateral Flow Test Strip.

A test is **invalid** if a Control Line (C) is missing, smeared, or uneven, or if either Test Line is uneven. It is invalid if the diluted extract is obscuring either the Control (C) or Test Line (T) or if the beads do not flow past Test and Control Lines. Any strip that does not develop a Control Line should be discarded. A second preparation of the extract (using a fresh dilution) should be made and tested using another strip.





g. Interpreting the Lateral Flow Test Strip using the ROSA-M Reader.

(1) Insert a clean valid test strip into the ROSA-M Reader. Slide the strip into the slot, with the sample compartment in the up position, until it stops.

LF-ROSA READER-M: ROSA-M Reader supplied with calibrators.

(2) Read result on **ZEAR** Channel (3-Line Mode) using the appropriate **MATRIX 00²** on the ROSA-M Reader. If desired, enter **Sample No.** and/or **Operator Name.** Press **ENTER** to read.

(3) **READING:** The number displayed is the concentration of Zearalenone (ppb) in the sample. Readings greater than 1000 ppb must be diluted and retested.

h. Equipment and supplies.

(1) Materials Supplied in Test Kits.

Kits can be purchased that contain 20, 100, or 500 strips and include Control and ZEAR Dilution Buffer.

(a) LF-Zearq-20:

1 Container of 20 zearalenone test strips.

2 Zearalenone Positive Control.

3 ZEAR Dilution Buffer.

(b) LF-ZEARQ-100:

1 Container of 100 zearalenone test strips.

2 Zearalenone Positive Control.

3 ZEAR Dilution Buffer.

(c) LF-ZEARQ-500:

1 5 containers of 100 zearalenone test strips.

2 5 Zearalenone Positive Controls.

3 5 ZEAR Dilution Buffer.

(2) Equipment/Materials required but not included in test kit:

(a) 1 ml REPIPET® or 1 ml Fixed Volume Pipette.

(b) 300 µl Fixed Volume Pipette.

(c) 100 µl Fixed Volume Pipette.

(d) 100 ml Graduated Cylinder.

(e) 1000 ml Graduated Cylinder.

(f) Micro-centrifuge Rack.

(3) Disposable Supplies.

(a) 1 L Storage Bottle.

(b) 200-1000 µl Pipette Tips.

(c) 20-200 µl Pipette Tips.

(d) 1.5 ml Micro-centrifuge Tubes.

(e) Specimen Containers.

(f) Large Weigh Dishes.

(g) 1 ml Syringes.

(h) FIL-0.45UM-RC15CS: Sartorius Minisart RC 15 Syringe Filters.(20 Box)

(4) Optional Equipment and Supplies.

(a) 110/220V: Mini-Centrifuge.

(b) 3 ml Disposable Transfer Pipettes.

(c) Filter Funnel.

(d) Filter Paper (Whatman 2V or equivalent).

(e) Rock-IT Shaker (available upon request).

i. Storage Conditions.

(1) Test Strips.

(a) Store refrigerated at 32 - 45° F in a tightly closed moisture container.

(b) Before use remove container from refrigerator and allow it to reach room temperature to limit condensation.

(c) Remove strips to be used for the day and return container to 32-45°F storage.

(d) Strips are stable at room temperature for at least 12 hours.

(e) If blue desiccant packets in container turn white or pink, performance test the strips with Negative and Positive Controls before continued use. Discard strips if invalid test results after performance test.

(2) Reagents.

(a) Negative & Positive Controls -Store at 32-45°F for up to 1 week, or aliquot and freeze at -4°F for up to 2 months.

(b) Zear Dilution Buffer.

1 Use at room temperature.

2 Micro-centrifuge tubes can be predispensed with 1.0 ml of ZEAR Dilution Buffer.

3 Store bottle and any unused predispensed tubes at 32 -45°F.

18. RIDASCREEN®FAST ZEARALENONE SC TEST KIT

Ridascreen® Fast zearalenone SC test is a competitive enzyme immunoassay (ELISA) for the quantitative analysis of zearalenone in select grains - corn & cracked corn only. **The test kit is limited to providing zearalenone measurements between 50 – 1000 ppb.**

a. Preparation of solutions.

(1) Extraction Solution.

The extraction solvent used in the Ridascreen® Fast Zearalenone SC test is a methanol/water (distilled or deionized) mixture consisting of 70 percent methanol (ACS grade or better) and 30 percent water.

(a) Using a graduated cylinder, measure 700 ml of methanol and place it into a clean carboy with spigot.

(b) Add 300 ml deionized or distilled water to the methanol and shake vigorously until it is completely mixed.

(c) Label the container stating the mixture (70 percent methanol and 30 percent water), date of preparation, and initials of technician who prepared the solution.

(d) Store this solution at room temperature in a tightly closed container until needed.

NOTE: To prepare smaller or larger amounts of solution use the ratio of 7 parts methanol to 3 parts of deionized or distilled water.

(2) Wash Solution.

Fill wash bottle with distilled/deionized water.

b. Extraction Procedures.

(1) Extraction procedure for Corn and Cracked Corn.

(a) Transfer 50-grams of ground sample into an extraction mixing jar.

(b) Add 250 ml of the (70/30) methanol/water extraction solvent.

(c) Cover the extraction jar and blend on high speed for 2 minutes.

(d) Filter the extract through a Whatman no. 1 filter or equivalent.

(e) Dilute 1 ml of the filtrate with 1 ml of distilled or deionized water.

(f) Proceed to test procedures.

c. Test procedures.

(1) Sample Analysis.

(a) Allow reagents, microwells, and sample extracts to reach room temperature prior to running any test. (20-25ºC, 68 - 77ºF)

(b) Insert a sufficient number of wells into the microwell holder for all standards and samples to be tested. (For example: to test 7 samples use 8 wells - 1 for the standard and 7 for the test samples).

Test Strip

Well #	1	2	3	4	5	6	7	8
Sample	C 0	S1	S2	S3	S4	S5	S6	S7

Where C 0 is the zero control, S1 is sample 1, S2 is sample 2, S3 is sample 3, etc.

NOTE: Do not run more than 3 strips (23 samples) per set of control standards.

(c) Using a new pipette tip for each standard and sample, pipet 50 µl of standard and prepared sample to separate wells.

(d) Add 50 µl of enzyme conjugate (red capped bottle) into each well.

(e) Add 50 µl of zearalenone antibody (black capped bottle) into each well.

(f) Mix thoroughly by gently sliding the microwell holder back and forth on a flat surface 10 - 15 seconds without spilling reagents.

(g) Incubate for 10 minutes (± 1.0 minutes) at room temperature (68 – 77º F).

(h) Dump the contents of the wells. Turn the wells upside down and tap out on a paper towel until the remaining liquid has been removed.

(i) Using a wash bottle, fill each well with distilled or deionized water. Empty the wells again and remove all remaining liquid. Repeat this step 2 times (total of 3 washes).

(j) Add 100 µl of substrate/chromogen (brown capped-brown plastic bottle) to each well.

(k) Mix thoroughly by gently sliding the microwell holder back and forth on a flat surface 10 - 15 seconds without spilling reagents.

(l) Incubate for 5 minutes (± 0.5 minutes) at room temperature (68 – 77º F). Cover the wells with a paper towel to protect them from light sources.

(m) Add 100 µl of stop solution (yellow capped--brown glass bottle) to each well.

(n) Mix thoroughly by gently sliding the microwell holder back and forth on a flat surface 10 -15 seconds without spilling reagents.

(o) Measure absorbance at 450 nm using the Biotek EL 301, or Awareness Technology Stat-Fax Model 303 PLUS microwell readers.

(p) Results must be read within 10 minutes.

d. Reading Results with the Microwell Reader.

(1) Biotek EL 301 Microwell Reader.

(a) Make sure that the microwell reader is on and allowed to warm-up for a minimum of 15 minutes before using.

(b) Remove sample carriage and hit "Enter."

(c) Insert W2 filter and hit "Enter."

(d) Insert W1 filter (450 nm) and hit "Enter."

(e) Hit "Clear" and then "Blank." This will cause the instrument to read air as the blank sample.

(f) Load microwells into sample carriage so that the first control labeled 0 is in position A1.

(g) Load the sample carriage into the strip reader so that position A1 is under the light beam of the reader.

(h) Press "Read" and an absorbance value for A1 should appear in the display on the microwell reader. Record the value.

(i) Slide the carriage to position A2 and press "Read." An absorbance value for A2 will appear. Record the value.

(j) Repeat step (i) until absorbance values have been obtained for the control and all samples. Record the values.

(k) Use the RIDA®SOFT Win Data software provided by r-Biopharm to convert the absorbance values into concentration values.

(2) Stat-Fax Model 303 PLUS Microwell Reader.

(a) To begin from the "Ready" prompt, press Menu, key in the test number, and then press Enter.

(b) The screen will read, "Set carrier to A, press enter." Place the wells all the way to the right in the carrier. Push the carrier all the way to the left to line up the notch with the wells, then press enter. The carrier will advance into the reader, and it should start to print.

(c) When the reader is finished reading the strip, the screen will read, "Plot Curve Y/N?"

Press "Yes" (1/A) to print the graph,

Press "No" (0) to skip this feature.

(d) The screen will read, "Accept Curve Y/N ?"

Press "Yes" (1/A) to accept the curve and proceed to read another strip. When finished reading the second strip, press "Clear" twice and the results strip will print, "Test Ended."

Press "No" (0) to end the test.

e. Equipment and Supplies.

(1) Materials Supplied in Test Kits.

(a) 1 microtiter plate.

(b) 48 antibody coated microwells.

(c) 1 Zearalenone standard solution of 1.3 ml of 0 ppb zearalenone solution.

(d) 1 red-capped bottle of 3 ml peroxidase conjugated zearalenone solution.

(e) 1 black-capped bottle of 3 ml anti-zearalenone antibody.

(f) 1 brown capped-brown plastic bottle of 6 ml substrate/chromogen.

(g) 1 yellow capped-brown glass bottle of 6 ml stop reagent.

(2) Materials Required but not Provided.

(a) Methanol - ACS grade or better.

(b) Deionized or Distilled Water.

(c) 250 ml graduated cylinder.

(d) 25 to 50 ml container.

(e) Whatman No. 1 filter paper, or equivalent.

(f) Sample collection tubes.

(g) Waring high-speed blender with a one liter jar, or equivalent.

(h) Sample grinder.

(i) Balance.

(j) Biotek EL 301 or an Awareness Technology Inc. Stat-Fax Model 303 Plus Microwell reader equipped with a 450-nm filter.

(k) Eppendorf Repipettor, or equivalent, and 2.5 ml syringes.

(l) 50 µl, 100 µl, and 1000 µl pipettor and pipette tips.

(m) Paper towels, Kaydry paper or equivalent absorbent material.

(n) Waste receptacle.

(o) Timer: 3 channel minimum.

(p) Waterproof marker, Sharpie or equivalent.

(q) Wash bottle.

f. Storage Conditions.

(1) Storage Conditions of Test Kits.

(a) The reagents supplied with the test kit can be used until the expiration date on the kit label when stored refrigerated at temperatures between 35° F and 46° F. **(DO NOT FREEZE)**

(b) Return any unused microwells to their original foil bag and reseal them together with the desiccant provided.

(c) The substrate/chromogen solution is light sensitive, therefore, avoid exposure to direct light.

(2) Indication of Instability or Deterioration of Reagents.

(a) Any bluish coloration of the red stained substrate/chromogen solution is indicative for deterioration and the reagent should be discarded.

(b) A value of less than 0.6 absorbance units for the zero standard may indicate deterioration of reagents.

(3) Test Kit Precautions:

(a) Return all test kit components to storage/refrigerator after use.

(b) Avoid prolonged intervals between working steps.

(c) Avoid direct sunlight during incubations. Covering the microwell holder is recommended.

(d) Do not use test kits after expiration date.

(e) Do not interchange individual reagents between test kits with different lot numbers.

19. CERTIFICATION

Applicants for service can request results reported on a quantitative or qualitative basis. Quantitative test results are reported to the nearest whole number in ppb, or nearest hundredth in parts per million (ppm).

Certify the results in ppb or ppm as requested by the applicant. To convert ppb to ppm, divide the ppb result by 1,000.

Results may be reported on a separate certificate, or on the certificate for grade at the applicant's option. When zearalenone is certified for export shipments, date the certificate the actual date loading was completed. For domestic shipments, date the certificate with the actual date that the results are received.

a. For qualitative service, certify results as being equal to or less than a threshold (e.g., 500 ppb, 0.50 ppm), or exceeding the threshold by using the following statements:

"Zearalenone exceeds (specified threshold) ppb/ppm."

"Zearalenone equal to or less than (specified threshold) ppb/ppm."

b. For quantitative service, certify test results between the lower (50 ppb) and the upper (1,000 ppb) quantification limits to the actual ppb/ppm.

"Zearalenone (record actual result) ppb/ppm."

Results below 50 ppb (0.05 ppm) or exceeding 1,000 ppb (1.0 ppm) are certified as less than or greater than (as applicable) according to the test kit conformance limits.

"Zearalenone does not exceed (50 ppb) (0.05 ppm)."

"Zearalenone exceeds (1,000 ppb) (1.0 ppm)."

/s/ John Giler

John Giler, Director
Field Management Division