Testing Trucklots of Barley and Wheat for Deoxynivalenol (DON)

Introduction

Deoxynivalenol (DON) is a toxin produced by fusarium fungi. DON occurs in barley and other feed grains when grown under certain climatic conditions. The quality of beer can be adversely affected when malting barley with significant concentrations of DON are used. Illnesses have been observed in livestock that have consumed feed grains containing high levels of DON concentrations. As a consequence, wheat and barley lots often receive price discounts when DON concentrations exceed certain levels.

Levels of DON are typically measured in the marketing channels with commercially available test kits. Test kits typically are based on enzyme linked immunosorbent assay (ELISA) technology. DON measurements are often made on each lot delivered to the market by producers. Due to the significant economic consequences, the accuracy and precision of DON measurements is of great concern. This document covers the testing procedures used by the Grain Inspection, Packers and Stockyards Administration (GIPSA) of the U.S. Department of Agriculture.

Backgound

Fungi are probably one of the most numerous plant families on earth. By definition they are plants that contain no chlorophyll (can grow in conditions of little or no natural light) and range from single cells to a body of branched hyphae (tubular filaments) that often produce fruiting bodies that form molds, mushrooms, smuts and yeasts. Instead of producing their own food, fungi absorb nutrients from either a living or dead host material. Mycotoxins are metabolites (by-products) of the growth of the molds.

Fusarium graminearum is the parent fungi of DON. Wheat and barley are the most commonly effected grain crops but the same fungus does infect corn. In the field, it shows up as a brown discoloration at the base of barley glumes, a pink to reddish mold on the glumes and kernels of the wheat heads and the tips of the ears of corn. Spores of the fungi can stay dormant on infected residues left on or in the soil.

The optimal temperature range for the DON mold is 70 to 85 F with moisture levels preferred to be greater than 20 percent. This particular fungi has two distinct growth cycles, with the mold growing during the warm temperatures of daytime, while the toxins are produced during the cooler temperatures of the night.

Sampling

The first step in DON analysis is obtaining a representative portion. Great care should be taken when sampling, since sampling error can be a significant source of variation.

Obtaining a representative sample from a lot of grain is an important and essential part of mycotoxin analysis. If the sample is not representative, the analysis result will not represent the true quality of the lot. In order for a sample to be considered representative, it must be:

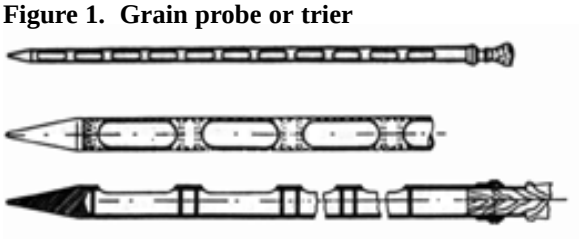
1. Obtained with equipment/procedures designed to obtain sample from all areas of the lot;
2. Of appropriate size;
3. Adequately identified;
4. Handled in such a way as to maintain representativeness.

Sampling Methods. A 1998 GIPSA study of ten trucklots of DON contaminated barley found evidence that some stratification of DON may occur. The lots in the study were from the 1997 harvest that were stored in farmer's bins over the winter. Some blending of the barley is likely to occur as the barley is moved from the field to farm storage and then to commercial elevator. Stratification may be more pronounced in lots coming directly from the field at harvest. Truck lots may be stratified in varying degrees depending on the non-uniformity in the field and harvesting practices.

To ensure that the sample that is tested is accurate, proper sampling techniques must be used to obtain a representative sample. A "coffee can" sample from the exposed layer of grain in a hopper car or truck, or a "bucket" sample as a truck or railcar is unloaded does not give a representative sample of the lot as a whole.

A large percentage of grain, as it travels from the farm to the final consumer, is sampled with a probe sometimes referred to as a trier. The probe is the only sampling method approved by GIPSA for stationary lots. If probe sampling is performed correctly, the samples drawn are considered representative.

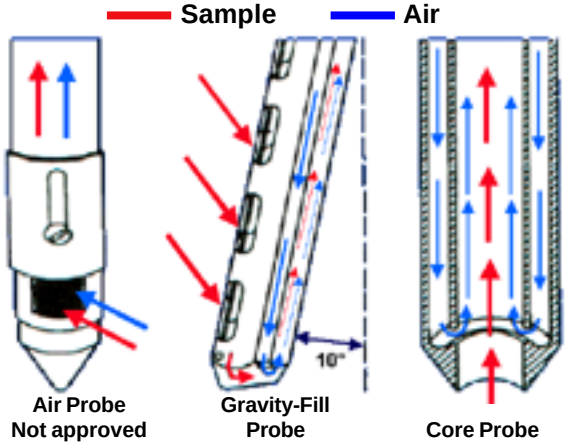
Figure 1. Grain probe or trier



Hand probes are constructed of brass or aluminum and come in various sizes with standard lengths of 5, 6, 8, 10, and 12 feet. The type of carrier and depth of grain dictates which probe length is used. For flatbed trucks or trailers use a 5 or 6 foot probe. For hopper bottom trailers 6, 8, or 10 foot probes are recommended.

Probe-type mechanical sampling systems have replaced hand probes at many facilities. GIPSA has approved two designs (gravity-fill and core) for probe-type mechanical samplers. In-load suction probes (air probe) are not approved because they draw air through the grain and vacuum excessive amounts of fine material into the sample.

Figure 2. Mechanical Probe Designs



Sampling patterns. How the sample is obtained with the probe will also affect the accuracy of results. A study conducted by Michigan State University found that the variability of DON measurements in trucks of newly harvested soft red winter wheat was significantly higher if less than four probes were taken from the lot.

GIPSA has established a sampling pattern for each type of carrier. The sampling patterns are designed to obtain a representative sample of approximately 2000-2500 grams which is more than adequate for DON analyisis.

The following diagrams indicate the standard sampling patterns. Insert the probe at the points marked, with the tip of the probe angled ten degrees in the direction of the arrow.

Figure 3. Seven probe pattern for flat-bottom trucks or trailers containing grain more than four feet deep.

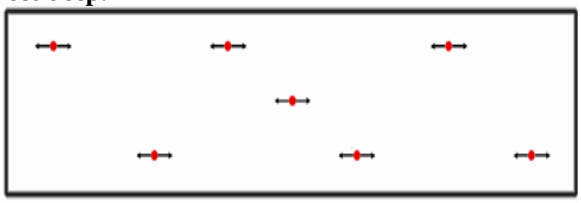


Figure 4. Nine probe pattern for flat-bottom trucks or trailers containing grain less than four feet deep.

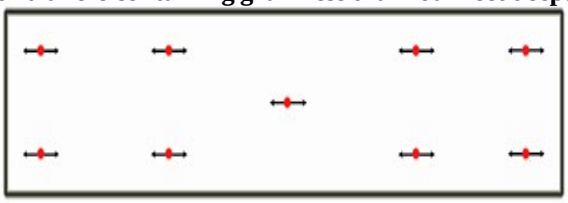
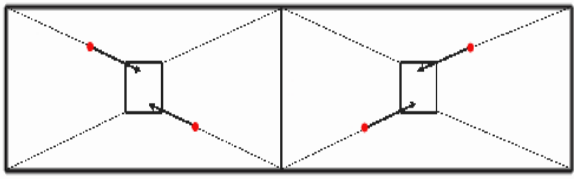


Figure 5. Four probe pattern for hopper bottom trailers or containers.



When two arrows are shown, the tip of the probe may be pointed in either of the indicated directions at the sampler's discretion.

Sample Preparation

The ground portion size required by GIPSA for barley and wheat is approximately 100 grams. A 1998 GIPSA study of DON contaminated barley has shown that increasing the size of the portion ground does not appear to significantly decrease the variability of DON results in barley. This does not mean that sample size is unimportant for DON analysis. As sample size is decreased below 100 grams, at some undetermined point, size would become a significant factor.

Clean the sample to remove dockage using a Carter dockage tester. The Carter dockage tester uses aspiration (air) and a combination of riddles and sieves to remove readily separable foreign material.

Photo 1. Carter dockage tester

Commercial/industry labs that do not have a Carter dockage tester can use hand sieves to remove dockage. For barley, use a 5/64 inch triangular-hole sieve. For wheat, use a 12/64 inch round-hole sieve on top of a 4.5/64 inch round-hole sieve. Pour the sample into the center of the top sieve, and shake twenty times. Hold the sieves and bottom pan level, and using a steady motion move the sieve from right to left about ten inches and return.

Photo 2. Handsieving

Consider dockage to be all coarse material that remains on top of the sieves and all material that passed throught he bottom sieve. Finally, hand pick the cleaned sample to remove any stones that might damage the grinder.

Photo 3. Boerner Divider

Use a Boerner divider, or a divider that gives equivalent results, to reduce the dockage free sample to two-100 gram portions. One of the 100 gram portions will be used for the test and the other will be set aside as a reserve. Do Not simply dip a portion from the probe sample as this can add significant sampling variability to the test. The Boerner divider reduces the size of a grain sample while maintaining the representativeness of the original sample.

Grind the sample using a Romer Mill, Bunn Model G3, Viking Hammermill, Falling Number Mill, UDY grinder or equivalent.

Photo 4. Romer Mill

Care should be taken to always use a clean grinding system. More care must be taken after grinding a contaminated sample than after a “clean” one. Some labs run a “clean” sample between lots for the purpose of cleaning the mill. Other labs run a few grams of the next sample and discard it to “purge” the mill. These practices are no guarantee that cross contamination will be eliminated. Only if the grinder can be completely opened up and cleaned can cross contamination between successive samples be avoided. The use of a

vacuum can make this procedure effortless. Dust creation should be kept to minimum whenever possible. Laboratories should never be dry swept but instead vacuumed with vacuums equipped with high efficiency particulate filters.

Grinding of samples should occur either in negative pressure rooms or in enclosed areas with exhaust hoods that will remove as much of the fine particulates as possible. Attaching a plastic bag to the outlet spout of the grinder will capture all of the sample without releasing dust into the air.

Once the ground sample is obtained, mix the sample by stirring with a spatula for thirty seconds or lifting or rolling the ends of the bag to the opposite side and repeating at least ten times. This will insure an even mix of particles. **This step is critcal for optimum results.** A 50 gram portion can now be removed and weighed.

Sample Analysis

Levels of DON are typically measured in the marketing channels with commercially available quick tests. GIPSA has approved the following three methods for quantitative DON analysis:

Neogen Veratox
Romer AccuTox
Romer FluoroQuant

The Neogen Veratox and Romer AccuTox test are based on enzyme linked immunosorbant assay (ELISA). The FluoroQuant test is based on fluorometry. The ELISA tests are the most common methods used for DON testing in the field (see attachments for more information).

Certification of Results

GIPSA has adopted procedures for reporting DON results that reflect the capability of current technology, meet the demands of domestic and export contracts that require stringent DON limits, and provide uniform certification results for buyers/sellers of U.S. grain. GIPSA approved the DON test kits with a quantitation range from 0.5 PPM to 5 PPM, and offers certification procedures that best describe the DON content.

GIPSA has tested and approved the current DON testing methods with a lower threshold of detection at 0.5 PPM, thus any measurements at or below 0.5 PPM are certified with the statement “Less than or equal to 0.5 PPM.” GIPSA’s ***standard*** certification procedures require that measurements at 0.6 PPM and above are rounded (according to the standard GIPSA rounding rules) and certified to the nearest whole number (e.g.,1 PPM, 2 PPM) in PPM. GIPSA’ standard method of certifying DON results is shown in table 2.

Table 1. GIPSA standard certification.

DON Test Results	Reported Value
0.0 to 0.5 PPM	Less than or equal to 0.5 PPM
0.6 TO 1.4 PPM	1 PPM
1.5 to 2.4 PPM	2 PPM
2.5 TO 3.4 PPM	3 PPM
3.5 TO 4.4 PPM	4 PPM
4.5 TO 5.0 PPM	5 PPM

GIPSA offers ***optional*** DON certification to the tenth PPM for lots that contain DON levels equal to or exceeding 0.6 PPM. GIPSA introduced this optional certification procedure to meet the demands of both domestic and export contracts that require stringent DON limits.

Variability

Any inspection result that GIPSA reports on an official certificate is deemed accurate (within a certain statistical variation) and is accepted by buyers and sellers of U.S. grain as a final result. Table 2 contains estimates of the repeatability observed when duplicate measurements are analyzed within a lab on the same sample extract. These estimates of repeatability are based on data from a 1998 GIPSA study of DON variability in Barley.

Table 2. Estimated Standard Deviation of duplicate analyisis of a sample extract within a lab.

DON Level	Barley StD
0.5 PPM	0.07
1 PPM	0.10
2 PPM	0.15
3 PPM	0.19
4 PPM	0.22
5 PPM	0.24

Table 3 contains estimates of the variability that can occur between multiple labs testing portions of the same ground sample. These estimates of variabilty are based on data from GIPSA check sample surveys conducted from 1993 to 1999.

Table 3. Estimated Standard Deviation of results from multiple labs testing the same ground sample.

DON Level	Wheat StD	Barley StD
0.5 PPM	0.32	0.24
1 PPM	0.45	0.31
2 PPM	0.63	0.39
3 PPM	0.76	0.46
4 PPM	0.87	0.51
5 PPM	0.97	0.56

Variability of measurements from an analytical process can be attributable to three primary sources: 1) the sample, 2) sample preparation, and 3) the analytical method. In DON analysis, no single source is clearly the dominant source of variation. The variation among measurements is the cumulative result of many steps in the process and may not be consistent from lot to lot. In addition, measurement variability will be significantly increased, if technicians are poorly trained, laboratory facilities are inadequate, or kit components are improperly stored.

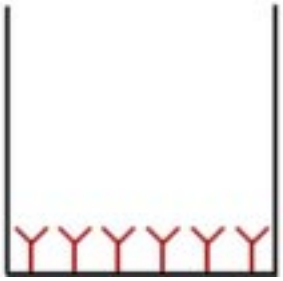
The keys to accurate and repeatable DON test results are as follows:

1. Obtain a representative sample.
2. Clean the sample to remove dockage.
3. Use a boerner divider to obtain a 100 g or larger representative sub-sample.
4. Grind subsample and mix thoroughly.
5. Purge or clean grinder between samples.
6. Use GIPSA approved methods and procedures (see attachments).
7. Maintain adequate laboratory facilities.
8. Only qualified technicians should run tests.

Neogen Veratox Method

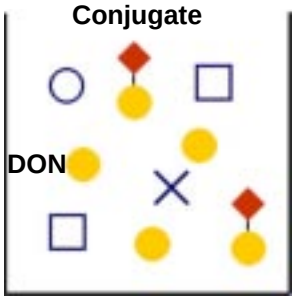
Overview. Neogen's "Veratox" (DON) uses ELISA technology. Antibodies specific for a mycotoxin are adhered to the inside of a microwell.

Figure 6. Neogen Microwell.



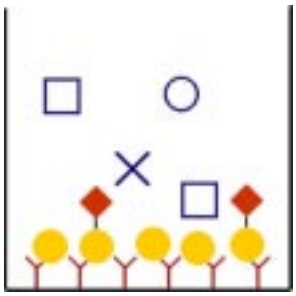
A solution of DON, chemically conjugated to an enzyme, is provided with the kit. A sample to be tested for DON is ground and extracted. The extract is then filtered and mixed with a fixed amount of the DON-enzyme conjugate solution in a mixing well.

Figure 7. Mixing well containing extract and conjugate.



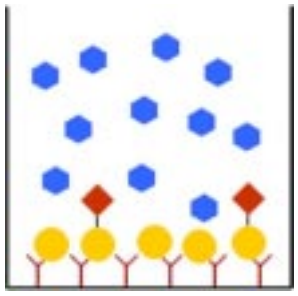
A portion of the mixture is then transferred to the antibody well. The DON from the extracted sample and DON-enzyme conjugate then compete for the antibody binding sites in the microwell.

Figure 8. Free toxin and conjugate compete for binding sites in microwell containing antibodies.



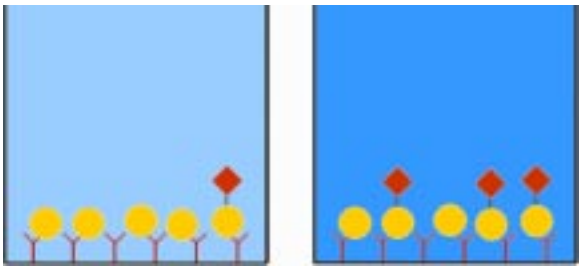
The assay procedure measures how much of the conjugate actually binds to the antibodies by first thoroughly washing the microwell then adding a colorless substrate.

Figure 9. After washing substrate is added.



The enzyme present in the microwell converts the substrate to a blue colored product; the more DON-enzyme conjugate in the microwell, the more intense the blue color.

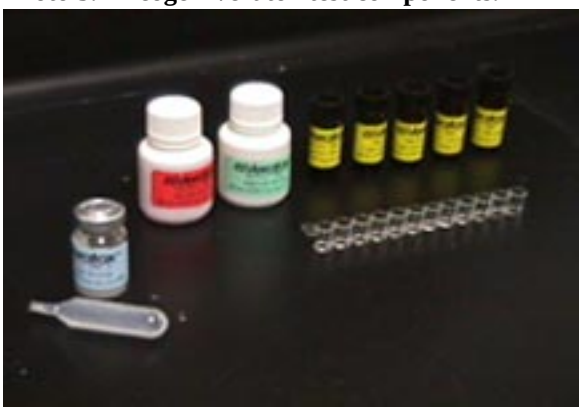
Figure 10. The conjugated enyme and substrate react to form a blue color.



Because samples with high DON will result in less binding of the DON- enzyme conjugate, positive samples will be lighter blue. Quantitative measurements are obtained by measuring the intensity of the color with an optical density reader.

Veratox Procedures. The Neogen Veratox DON test is provided as a kit containing all required reagents, controls and microwells.

Photo 5. Neogen Veratox test components.



NOTE: When not in use, kits should be stored in the dark at refrigeration temperatures. Prior to use, all necessary kit components must be equilibrated to room temperature. The use of cold reagents could adversely affect the color development of the ELISA test.

The kit components are as follows:

-Foil pouch with Antibody-coated well strips and red marked mixing well strips.

-Substrate. The substrate is pre-activated and ready for use.

-Conjugate. Unseal one of the conjugate bottles and remove the rubber stopper. Cut the tip off the enclosed squeeze tube. Squeeze tube contents into the bottle. Replace the stopper and swirl, don't shake, contents until the pellet has dissolved. Use the contents of the bottle until empty.

NOTE: Once rehydrated, contents must be used within 3 weeks. Mix the second bottle of conjugate in the same manner when needed. Refrigerate when not in use.

-Controls. The kit comes with 6 controls (0, 0.5, 1, 2, 4, and 6 ppm). The 4ppm control is not used in the official procedure.

-Red Stop. Swirl to mix prior to use.

TIP: Swirl, don't shake all reagents prior to use so as to mix them but not cause them to foam, which could cause pipetting errors later.

Step 1: Thoroughly mix the ground sample and weigh out 50 grams. Place the ground sample in an 18 ounce nasco whirlpack bag or clean plastic or glass container.

Step 2: Add 250 ml of distilled or deionized water. **Do not use tap water!** The pH of tap water may adversely effect results.

Photo 6. Adding distilled water.

Step 3: Shake mechanically or by hand for 3 minutes. Let the material stand for 1-2 minutes to let some of the sample settle.

Step 4: Place a sheet of filter paper (Whatman #1 folded or equivalent) into a clean funnel mounted over a test tube or collection beaker. After much of the sample slurry has settled to the bottom of the bag, pour at least 15 mL of the extract through the filter paper.

Photo 7. Filter Extract.

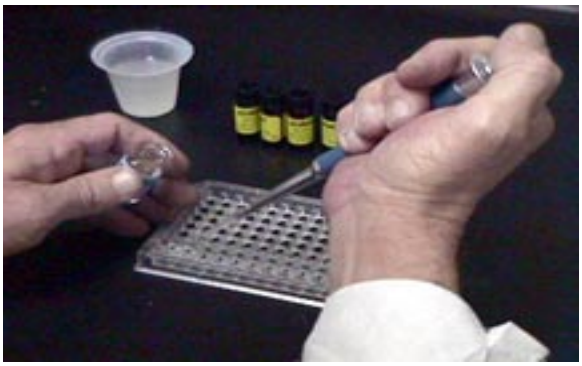
Step 5: Remove a red-marked mixing well strip and break off the needed number of wells-one for each control and one well for each sample (up to seven). Return unused strips to package.

Remove an antibody-coated well strip and break off the same number of wells. Mark one end of the antibody-well strip with a 0 (zero) for the blank and the other end with an S for samples and place strip in the well holder with the 0 marked end on the left.

NOTE: Do not mark the inside or bottom of the antibody wells.

Step 6: Firmly place a pipette tip on the 100µl pipettor and add 100µl conjugate to each mixing well. Discard tip.

Photo 8. Add conjugate to each mixing well.



TIP: Prime pipette tips before dispensing conjugate. To prime a tip, draw up some of the reagent to be dispensed and discharge it back into the same container. Priming coats the inside of the pipette tip so that the volume dispensed will be identical during repeated use of the same tip.

Step 7: Remove the stopper from the 0 ppm control bottle. Using a new pipette tip, add 100µl of the 0 ppm control to the first mixing well (labeled 0). Discard tip and replace cap on control bottle. Repeat with the 0.5, 1.0, 2.0, & 6.0 ppm control bottles, using a clean tip for each control.

Add 100 µL of filtrate from the sample collection tube of the first sample to the sixth well. Discard tip. Repeat for each sample, placing 100µl from each in a different well. Use a new tip for each sample.

TIP: When drawing reagents into a pipette tip, drag the tip across the rim of the reagent bottle to remove any excess liquid.

TIP: When dispensing reagents into the microwells, place the tip point against the inside wall of the microwell. This helps draw all of the liquid out of the tip and eliminates drops that form on the end of the tip.

TIP: Always check the fluid levels in your tips prior to dispensing to be sure that the same amount is being collected each time. If the proper amount was not collected, or bubbles are present, refill the tip.

Step 8: Firmly place pipette tips onto the 12-channel pipettor. Mix the solutions by depressing the plunger 5 times. Transfer the solutions to the marked antibody wells. Discard the red marked mixing wells.

Photo 9. 12-channel pipettor.

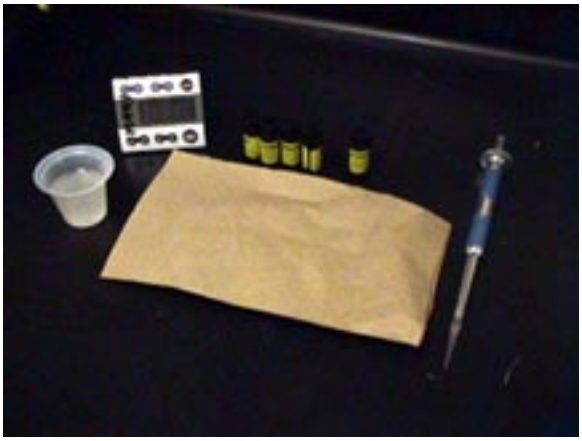
Step 9: Mix by sliding the Microwell holder back and forth on a flat surface to ensure mixing (15-20 seconds). Do not splash reagents from wells.

Step 10: Cover the wells to protect them from dust and allow them to incubate for 10 minutes.

Photo 10. Mix solutions.

During this incubation, the conjugated DON and any free DON from the sample or standard will compete for binding sites in the antibody wells. The more free DON in solution, the less conjugate will be bound to the antibody well.

Photo 11. Cover the wells during incubation.



Step 11: Initial reaction is now complete. Shake out the contents of antibody-coated wells. Using a wash bottle, fill each antibody-coated well with distilled water and shake out. Repeat five times. Any bound conjugate or DON will not be removed during the wash steps.

Step 12: Remove all large water droplets by turning wells upside down and vigorously tapping wells on paper towel. Bound conjugate or DON will not be removed by the tapping.

TIP: To remove water droplets, wrap the well holder in a paper towel and rap the wells vigorously five or six times or until no fresh water droplets appear on the paper towel. When the water has been removed, loosen the wells in the well holder so they will be easier to remove after the substrate and red stop have been added.

Step 13: Pipette 3 mL of substrate into reagent boat and, with new tips on the 12-channel pipettor, place 100µl substrate into the wells.

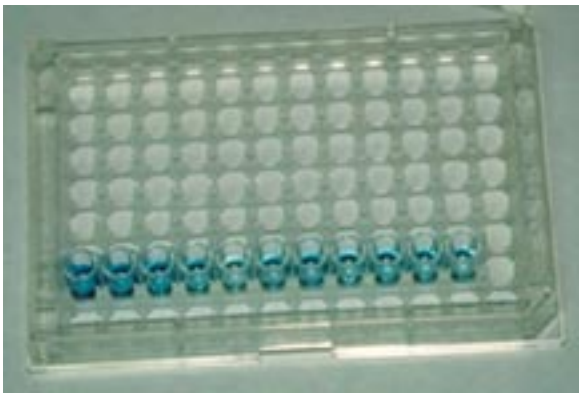
Photo 12. Removing water droplets from wells.

Step 14: Mix by sliding wells back and forth on flat surface. Cover the wells to protect them from dust. Incubate 10 minutes.

During the 2nd incubation, color development will begin. The substrate will react with the DON-conjugated enzyme to change the reagent color to blue. Less toxin bound means more conjugate present, and the darker the blue color.

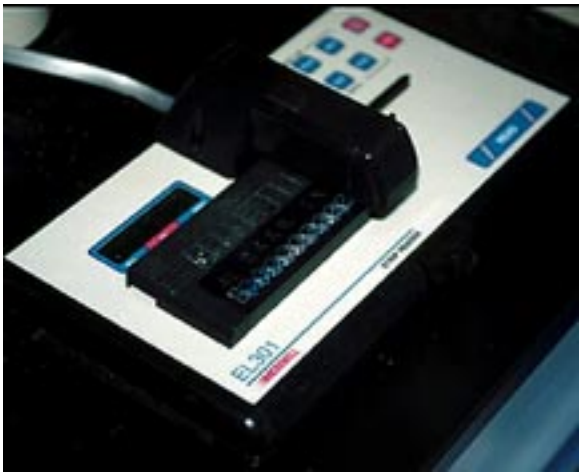
Step 15: Pipette 3 mL of red stop solution into a clean reagent boat. Using the 12-channel pipettor add 100µl of red stop to each well and mix as before. This will halt the color development.

Photo 13. Color development.



Step 16: The optical density (amount of color) of each well is read on a microwell reader. The optical density (OD) values of the controls are entered into the Log/Logit program used to develop a calibration curve.

Photo 14. EL 301 microwell reader.

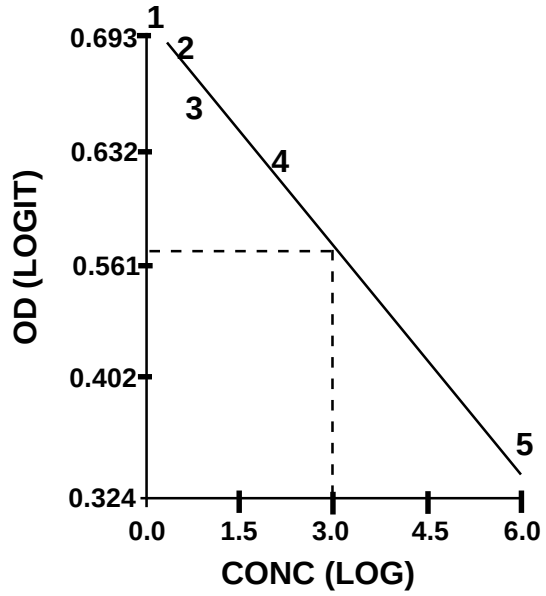


NOTE: Neogen also offers an automated microwell reader (Stat Fax 303 Plus). Contact your Neogen representative for information.

TIP: For optimum results the OD on the zero standard should be 0.6 or higher. Cool laboratory temperatures (below 72 F) may slow the reactions requiring a longer incubation period. If your OD on the zero standard is less than 0.6 try lengthening the incubation time to 11 or 12 minutes.

The Log/Logit program provided by Neogen first normalizes the data to create a linear (straight line) relationship. It then performs a linear regression to determine the straightline that best fits the control data.

Figure 11. Veratox calibration



The program reports two statistical values which are used to determine if the calibration is acceptable. The **correlation coefficient** values, or "r", indicate how well the five control samples fit the line. If all five of the values fall exactly on the line the correlation is perfect (1.0). The correlation must read 0.98 or higher to ensure accurate results. If correlation value is less than 0.98, rerun the test.

The **slope** value indicates how steep the line is angled. The slope value must read –2.0, +/- 0.5.

NOTE: Do not certify results if the correlation coefficient is less than 0.98 or the slope value is out of tolerance. If the slope value consistently reads outside these tolerances, or if the correlation coefficient is consistently below 0.98 call Neogen (1-800-234-5333) for troubleshooting assistance .

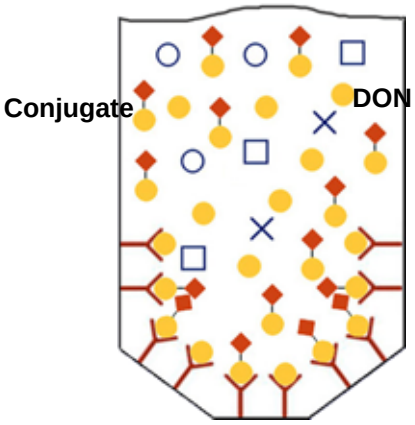
Romer Accutox Method

Overview. Romer Lab's "AccuTox" test for deoxynivalenol is an ELISA method. Antibodies specific for a mycotoxin are adhered to the bottom of a tube.

Figure 12. Romer Antibody Tube.

A sample to be tested for DON is ground, extracted with distilled water, and filtered. A solution of DON, chemically conjugated to an enzyme, is provided with the kit. The filtered extract is mixed with an equal amount of the DON-enzyme conjugate in an antibody tube.

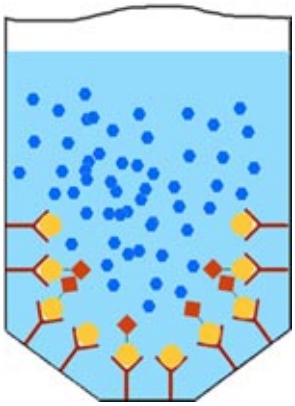
Figure 13. Free toxin and conjugate compete for binding sites in microwell containing antibodies.



After the toxin and conjugate have had time to attach to the antibodies, the remaining solution is discarded and the tube rinsed with Wash Solution.

After tapping the tubes on a paper towel to remove the water, substrate solution is added. The conjugated enzyme present causes the substrate to turn blue. The more conjugate, the more intense the color.

Figure 14. After washing. substrate is added which reacts with the conjugated enzyme to form a blue color.



After allowing the color change to develop for five minutes, "Stop Solution" is added. This stops the reaction and changes the color of the solution to yellow.

Quantitative measurements are obtained by measuring the intensity of the color with a Hach spectrophotometer. Because samples with high DON levels will result in less binding of the conjugate, positive samples will be lighter color.

Figure 15. The solution turns yellow when the stop solution is added.

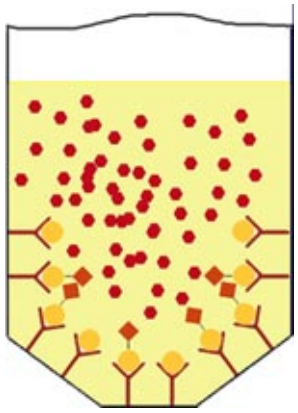


Photo 15. Accutox kit components.

Step 1: Thoroughly mix the ground sample and weigh out 50 grams. Place the ground sample in an 18 ounce nasco whirlpack bag or clean plastic or glass container.

Photo 16. Weighing 50 gram portion.

Step 2: Add 250 ml of distilled or deionized water. **Do not use tap water!** The pH of tap water may adversely effect results.

Step 3: Shake mechanically or by hand for 3 minutes. Let the material stand for 1-2 minutes to let some of the sample settle.

Step 4: Place a sheet of filter paper (Whatman #1 folded, S&S 24-cm pleated, or equivalent) into a clean funnel mounted over a test tube

<u>AccuTox Procedures</u>. The Romer Accutox DON test is provided as a kit containing all required reagents, controls and antibody tubes

NOTE: When not in use, kits should be stored in the dark at refrigeration temperatures. Prior to use, all necessary kit components must be equilibrated to room temperature (approximately 1 hour). The use of cold reagents could adversely affect the color development of the ELISA test.

The kit components are as follows:

-Antibody coated tubes in zip-lock bag
-Substrate.
-Enzyme Conjugate.
-Zero Calibrator Standard
-Control Solution
-Stop Solution.
-Wash solution. Store at room temperature

TIP: Swirl, don't shake all reagents prior to use so as to mix them but not cause them to foam, which could cause pipetting errors later.

Photo 17. Grain sizer used to shake DON samples.

or collection beaker. After much of the sample slurry has settled to the bottom of the bag, pour at least 15 mL of the extract through the filter paper.

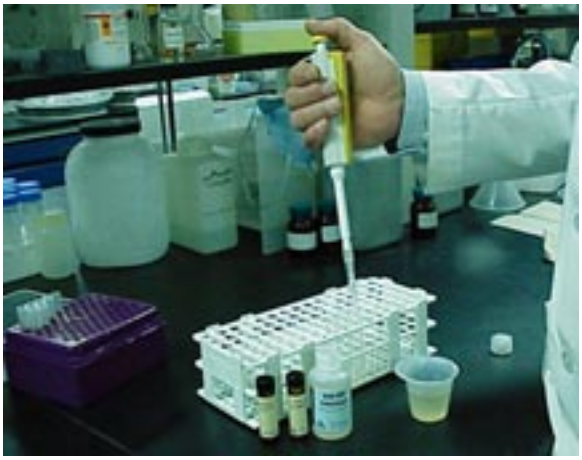
Step 5: Place the appropriate number of labelled anti-body coated tubes into gripper tube rack.

Photo 18. Place tubes in gripper rack.

NOTE: Do not exceed 30 tubes per run. Be sure to re-seal unused tubes in zip-lock bag with desiccant.

Step 6: Pipet 0.5 ml of zero calibrator into appropriate antibody-coated tube and discard tip. repeat this step for the control and filtered sample extracts .

Photo 19. Adding zero calibrator, control, and sample extracts to tubes.



Step 7: Pipet 0.5 ml of Enzyme Conjugate into each tube and incubate for 15 minutes.

NOTE: Start timing for 15 minutes as soon as conjugate has been added to first tube.

Photo 20. Incubate for 15 minutes.

TIP: Prime pipette tips before dispensing conjugate. To prime a tip, draw up a pre-measured amount of the reagent to be dispensed and discharge it back into the same container. Priming coats the inside of the pipette tip so that the volume dispensed will be identical during repeated use of the same tip.

Step 8: Mix contents of tubes by shaking rack vigorously for 5 seconds.

During this incubation, the conjugated DON and any free DON from the sample or standard will compete for binding sites on the antibody tube. The more free DON in solution, the less conjugate will be bound to the antibody well.

Step 9: At the completion of the 15 minute incubation dump contents of tubes into appropriate waste container. (e.g. sink, bucket, etc.). Fill tubes with Wash Solution and dump wash. Repeat three times for a total of four washes.

Photo 21. Wash tubes four times.

NOTE: It is very important not to underwash tubes. Overwashing will not affect the test.

Step 10: Following last wash, tap inverted tubes onto absorbant paper several times to remove all wash solution.

NOTE: It is very important to rid tubes of as much wash as possible.

Step 11: Pipet 0.5 ml of substrate into each tube and incubate or 5 minutes.

Photo 22. Tapping tubes on paper towel to remove wash solution.

NOTE: Start timing for 5 minutes as soon as substrate has been added to first tube.

NOTE: Solutions should all turn blue after substrate has been added.

Photo 23. Five minute incubation. Solutions turn blue after substrate is added.

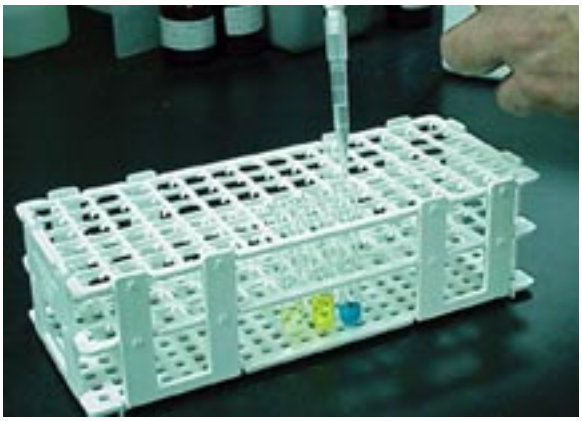
Step 12: Mix contents of tubes by shaking rack vigorously for 5 seconds.

Step 13: At the completion of the 5 minute incubation, pipet 0.5 ml of Stop Solution into each test tube.

Step 14: Mix contents of tubes by shaking rack vigorously for 5 seconds.

NOTE: Solutions should all turn yellow after adding Stop Solution.

Photo 24. Solutions turn yellow when the Stop solution is added.



Step 15: With the spectrophotometer set at 450 nm, blank with a clean unscratched test tube filled with fresh distilled or deionized water.

Step 16: Dry each tube with a paper towel before inserting into spectrophotometer. read and record absorbances of the calibrator, control, and samples.

Photo 25. Hach Spectrophotometer.

Step 17: Calculate results using log/logit data computer program with the factory calibration included with the kit.

NOTE: The control must fall within the acceptable range listed on the control vial. If not, troubleshoot and repeat the run. Call Romer Labs Technical Services (1-800-769-1380) for troubleshooting assistance.