

10.1 GENERAL INFORMATION

The Veratox AST test is a quick diagnostic tool to predict the presence of aflatoxin in corn and other commodities. The kit uses an enzyme-linked immunosorbent assay (ELISA) technique to obtain quantitative results from absorbance readings at 650 nm when sample readings are compared to a 20 ppb control and a pre-generated standard curve (0 to 400 ppb).

10.2 PREPARATION OF EXTRACTION SOLUTION

The extraction solvent used in the Veratox test method 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.

10.3 EXTRACTION PROCEDURES

- a. Place a sheet of filter paper (Whatman 2V folded or S&S 24 cm pleated or equivalent) into a clean funnel mounted over a 25 X 200 mm (diameter x length) test tube or a collection beaker.
- b. Label the collection container with the sample identification.
- c. Place the 50-gram portion of the ground sample into the blender container.

- d. Pour in 250 ml of the 70/30 percent methanol/water solution and securely close the blender top.
- e. Blend for exactly two minutes at high speed.
- f. Pour the resultant mixture from the blender into the funnel containing the filter paper and collect approximately 25 ml of extract.

10.4 TEST PROCEDURES

a. Preparation of Solutions.

- (1) Place 3 ml of substrate (light green labeled bottle) solution into a clean, labeled reagent boat. Cover boat to protect solution from dust and light.

NOTE: Do not return any substrate solution to the original bottle once it has been removed.

- (2) Place 3 ml of Red Stop (red labeled bottle) solution into a clean reagent boat. Cover boat to protect solution from dust and light.

b. Sample Analysis.

Do not use reagents or microwells from one kit serial number with reagents/wells from a different serial number. Reagent boats may be rinsed and reused.

- (1) Open foil bag and remove 3 red-marked mixing wells for each sample to be tested (maximum of 4 samples or 12 wells). Place them in the microwell holder, and mark the left end of each strip with a "1."

Do not run more than four samples at one time.

- (2) Remove 3 antibody wells for each sample to be tested (maximum of 4 samples or 12 wells). Place them in the microwell holder, and mark the left end of each strip with a "1."
- (3) Reseal bag by folding over and tightly closing with a suitable fastener (large paper clip, tape, or suitable dust and light protectant).

- (4) Place 100 µl of conjugate (blue-labeled bottle) into each mixing well using a 100 µl pipettor with a new tip. Prime the pipette tip first before dispensing the 100 µl. Discard the pipette tip.

NOTE: "Prime the pipette tip" is accomplished by drawing liquid up into the tip and dispensing it back into the bottle once or twice.

- (5) Place 100 µl of control (yellow-labeled bottle) into the first mixing well labeled "1." Prime the tip before dispensing. If testing more than one sample, also place 100 µl of control into mixing well #4 for the second sample, mixing well #7 for the third sample, and mixing well #10 for the fourth sample. Discard the pipette tip.
- (6) Place 100 µl of sample each in mixing wells #2 and #3. Prime the tip first before dispensing. Discard the tip. Subsequent samples should be placed in wells #5 and #6, then #8 and #9, and then #11 and #12.

See the diagram below for an example of the procedure.

mixing wells	W1	W2	W3	W4	W5	W6	W7	W8	W9	W10	W11	W12
	O	O	O	O	O	O	O	O	O	O	O	O
	C	S1	S1	C	S2	S2	C	S3	S3	C	S4	S4

"W" = well number (e.g., #1 through #12)

"C" = control

"S1, S2, S3, & S4" = sample numbers

- (7) Using the 12 channel pipettor and the overfill method (see note below), mix the contents of the mixing wells by pipetting up and down in the tips 5 times.

NOTE: The "overfill method" is performed by drawing greater than 100 µl into the pipette tips by pressing the pipettor to the second stop before placing tips into the solution. Place tips into the liquid and release the plunger slowly and completely.

- (8) Transfer 100 μ l to the antibody coated wells (the unmarked, clear wells). To dispense only 100 μ l, press plunger to the first stop.
- (9) Mix in the antibody coated wells by gently sliding the microwell holder back and forth on a horizontal surface for 15 seconds. Be careful not to allow solution to splash out of wells.
- (10) Immediately following mixing, incubate for 5 minutes. Discard all mixing (red marked) wells and tips.
- (11) With a wash bottle containing deionized/distilled water, fill each antibody well and dump the contents into a waste receptacle. Repeat this step five times.
- (12) Turn microwell holder, with wells in it, upside down on a paper towel and tap gently until water is removed from the wells.
- (13) Using the 12 channel pipettor and the overfill method, place 100 μ l of substrate into each well.
- (14) Mix gently by sliding the microwell holder back and forth for 15 seconds on a horizontal surface for 15 seconds. Be careful not to allow solution to splash out of wells.
- (15) Immediately following mixing, incubate for 5 minutes.
- (16) Discharge the remaining substrate in the pipette tips by plunging once or twice without drawing any additional liquid up into the tips. Save these tips for the next step.
- (17) Using the 12 channel pipettor and the overfill method, add 100 μ l of the red stop solution (red labeled bottle) into each well.
- (18) Mix gently by sliding the microwell holder back and forth for 15 seconds. Again be careful not to lose any solution from the wells. Visually check the appearance of the wells. Discard all pipette tips.
- (19) Read in a microwell reader using a 650 nm filter within 5 minutes of the addition of the red stop solution.

If the OD readings for the duplicate portions both indicate that the aflatoxin content exceeds 300 ppb but a "CV overrange" message appears, then:

- (1) an additional sample portion (diluted) must be tested until the OD values of duplicate portions are within 15 percent of the mean OD,

or

- (2) official personnel may stop the testing if the applicant requests only a certification statement that aflatoxin exceeds 300 ppb. In this instance, official personnel will certify the aflatoxin testing results as a "Aflatoxin exceeds 300 ppb."

10.5 REPORTING AND CERTIFYING TEST RESULTS

- a. Report all results on the pan ticket and the inspection log to the nearest whole ppb.
- b. Sample results over 300 ppb are reported as >300 ppb unless a supplemental analysis is performed.
- c. Refer to the Certification section of the handbook for more detailed certification procedures.

10.6 SUPPLEMENTAL ANALYSIS

- a. Diluting the Sample Extract.

If quantitative results are above the test method's conformance limit, test results are reported as exceeding the limit. To determine and report an aflatoxin level higher than 300 ppb, the sample extract must be diluted so that a value between 5 and 300 ppb is obtained.

The final aflatoxin concentration is calculated by multiplying the results with the diluted extract by the dilution factor.

b. Example.

If the original analysis reported the aflatoxin value at 700 ppb, the sample extract would be diluted using the following procedures in order to obtain a true value.

- (1) Dilute 5 ml of the original extract with 10 ml of the extraction solvent mixture. The total volume is 15 ml. This is a 1 to 3 dilution (compares volume in the beginning with the total volume in the end).
- (2) Multiply the analytical results obtained by 3 to obtain the actual aflatoxin concentration. For example, if 240 ppb was the original value obtained with the diluted extract, the actual concentration in the original sample was 720 ppb.

$$\text{True Aflatoxin Value} = \frac{\text{Total Volume}}{\text{Initial Extract Volume}} \times \text{Aflatoxin Result}$$

$$\begin{aligned} \text{True Aflatoxin Value} &= (15 \div 5) \times 240 \text{ ppb} \\ &= 3 \times 240 \text{ ppb} = 720 \text{ ppb} \end{aligned}$$

10.7 CLEANING LABWARE

a. Negative Tests (≤ 20 ppb).

(1) 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.

(2) Disposable Materials.

Place materials in a garbage bag for routine trash disposal.

CHAPTER 11

MYCO✓ TEST KIT

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11.1 GENERAL INFORMATION

The Myco✓ test is a competitive enzyme-linked immunosorbent assay that provides quantitative measurement for the presence of aflatoxin in corn, corn meal, corn soy blend sorghum and popcorn.

11.2 PREPARATION OF SOLUTIONS

a. Extraction Solution.

The extraction solvent used in the Myco✓ test method is a methanol/water (distilled or deionized) mixture consisting of 70 percent methanol (ACS 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. Wash Solution.

- (1) Transfer the contents of the Wash Concentrate vial to a 500-ml plastic squeeze bottle and add 475 ml of distilled or deionized water.
- (2) Swirl to mix.

11.3 EXTRACTION PROCEDURES

- a. Place a sheet of filter paper (Whatman #1 folded or equivalent) into a funnel mounted over a clean collection container.
- b. Label the collection container with the sample identification.
- c. Transfer 50 grams of ground sample into an extraction mixing jar.
- d. Add 250 ml of the (70/30) methanol/water extraction solvent.
- e. Cover the extraction jar and blend on high speed for 2 minutes.
- f. Allow the extract to stand for 2-3 minutes to allow the slurry to settle.
- g. Filter a minimum of 15 ml of the extract into the collection container.

11.4 TEST PROCEDURES

- a. Allow reagents, antibody-coated wells, mixing wells, and sample extracts to reach room temperature prior to running the test.
- b. Place the appropriate number of red mixing wells and clear test wells into a microwell holder.

NOTE: The maximum number of test samples that can be run at one time is 19. Using a strip of 12 wells, designate 5 wells for the calibrators and the remainder of the wells for test samples.

- c. Using a pipette, dispense 150 μ l of Enzyme Conjugate into each red mixing well.
- d. Dispense 50 μ l of each calibrator and sample into the appropriate red mixing wells using an adjustable or fixed 50 μ l pipette.

NOTE: Use a clean pipette tip for each addition.

mixing wells	W1	W2	W3	W4	W5	W6	W7	W8	W9	W10	W11	W12
	O	O	O	O	O	O	O	O	O	O	O	O
	C0	C10	C20	C40	C80	S1	S2	S3	S4	S5	S6	S7

Where C0 is the zero control, C10 is the 10 ppb control, C20 is the 20 ppb control, C40 is the 40 ppb control, and C80 is the 80 ppb control. S1 is sample 1, S2 is sample 2, S3 is sample 3, etc.

- e. Using a multi-channel pipette, mix the contents of the wells by repeatedly filling and emptying the tips into the mixing wells.
- f. Using a multi-channel pipette, transfer 100 µl of each reaction mixture directly into the corresponding clear test wells. Discard the mixing wells into an appropriate waste container.
- g. Let the reaction mixture incubate for **exactly 5 minutes**. Mix the solution in the wells by gently swirling the plate on a flat surface for the first 15 seconds.
- h. At the end of the 5-minute incubation period, dump the contents of the wells into an appropriate waste container. Using a 500-ml squeeze bottle containing wash solution, vigorously wash each well by overfilling. Repeat the vigorous wash for a **total of four washes**.
- i. After the last wash, invert the wells and tap on absorbent paper to remove residual wash solution. Wipe excess liquid from the bottom of the wells.
- j. Pour substrate solution into a clean reagent reservoir.
- k. Dispense 100 µl of substrate solution into each test well using a multi-channel pipette.
- l. Let the substrate solution incubate for **exactly 5 minutes**. Mix the solution in the wells by gently swirling the plate on a flat surface for the first 15 seconds.
- m. Pour stop solution into a clean reagent reservoir.

- n. Dispense 100 µl of stop solution into each test well using a multi-channel pipette.
- o. Read and record the optical density of the wells at 650 nm using a Hyperion MicroReader™ 3 well reader. Make sure that the well bottoms are clean and dry before placing in the reader. Read the test results within 20 minutes of test completion. Use the data reduction software provided by SDI to quantify results.

11.5 REPORTING AND CERTIFYING TEST RESULTS

- a. Report all results on the pan ticket and the inspection log to the nearest whole ppb.
- b. Sample results over 300 ppb are reported as >300 ppb unless a supplemental analysis is performed.
- c. Refer to the Certification section of the handbook for more detailed certification procedures.

11.6 SUPPLEMENTAL ANALYSIS

- a. Diluting the Sample Extract.

If quantitative results are above the test method's conformance limit, test results are reported as exceeding the limit. To determine and report an aflatoxin level higher than 300 ppb, the sample extract must be diluted so that a value between 5 and 300 ppb is obtained.

The final aflatoxin concentration is calculated by multiplying the results with the diluted extract by the dilution factor.

- b. Example.

If the original analysis reported the aflatoxin value at 700 ppb, the sample extract would be diluted using the following procedures in order to obtain a true value.

- (1) Dilute 5 ml of the original extract with 10 ml of the extraction solvent mixture. The total volume is 15 ml. This is a 1 to 3 dilution (compares volume in the beginning with the total volume in the end).

- (2) Multiply the analytical results obtained by 3 to obtain the actual aflatoxin concentration. For example, if 240 ppb was the original value obtained with the diluted extract, the actual concentration in the original sample was 720 ppb.

$$\text{True Aflatoxin Value} = \frac{\text{Total Volume}}{\text{Initial Extract Volume}} \times \text{Aflatoxin Result}$$

$$\begin{aligned}\text{True Aflatoxin Value} &= (15 \div 5) \times 240 \text{ ppb} \\ &= 3 \times 240 \text{ ppb} = 720 \text{ ppb}\end{aligned}$$

11.7 CLEANING LABWARE

a. Negative Tests (≤ 20 ppb).

(1) 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.

(2) Disposable Materials.

Place materials in a garbage bag for routine trash disposal.

b. Positive Tests (> 20 ppb).

(1) Labware.

Prepare a bleach solution consisting of 1 part bleach to 10 parts water (e.g., 100 ml bleach to 1,000 ml water). Completely submerge the used glassware, funnels, beakers, etc., and soak for at least 5 minutes. Remove items from the bleach/water solution, submerge in a dishwashing liquid/water solution, wash thoroughly, then rinse with clean water before reusing.

(2) Disposable Materials.

Prepare a bleach solution consisting of 1 part bleach to 10 parts water in a plastic pail labeled "bleach solution". Soak disposable materials, such as used columns, cuvettes, vials, test kit components, etc., for at least 5 minutes. Pour off the liquid down the drain and place the materials in a garbage bag and discard.

11.8 WASTE DISPOSAL

a. Negative Results (≤ 20 ppb).

If the test result is negative (equal to or less than 20 ppb), discard the filter paper and its contents (ground material) into a plastic garbage bag for disposal. Dispose of any remaining liquid filtrate in the chemical waste container.

b. Positive Results (> 20 ppb).

If the result is positive (more than 20 ppb), the ground portion remaining in the filter paper must be decontaminated prior to disposal. After disposing of the remaining filtered extract in the chemical waste container, filter approximately 50 ml of bleach through the filter containing the ground portion and allow to drain. Discard the filter paper and its contents (ground portion) into a plastic garbage bag for disposal. The bleach rinse filtrate collected may be treated as a non-hazardous solution and disposed of by pouring down the drain.

11.9 EQUIPMENT AND SUPPLIES

a. Materials Supplied in Test Kits.

- (1) 48 antibody-coated microtiter wells.
- (2) 48 red-marked mixing wells.
- (3) 5 vials each containing 2 ml of 0, 10, 20, 40, and 80 ppb of aflatoxin calibrators.
- (4) 1 vial containing 8 ml of aflatoxin-HRP enzyme conjugate.
- (5) 1 vial containing 8 ml of substrate.

- (6) 1 vial containing 8 ml of stop solution.
- (7) 1 vial containing 25ml of 20X wash concentrate.
- (8) 4 multi-channel pipette reservoirs.

b. Materials Required but not Provided:

- (1) Methanol - ACS grade or better.
- (2) Deionized or distilled water.
- (3) 100 ml graduated cylinder.
- (4) Whatman #1 filter paper or equivalent.
- (5) Glassware with 125 ml capacity for sample extraction.
- (6) Filter funnel.
- (7) 50 μ l pipette with disposable tips.
- (8) 50 -200 μ l multi-channel pipette.
- (9) 500 ml plastic squeeze bottle.
- (10) Blender with mixing jars.
- (11) Balance.
- (12) Sample grinder.
- (13) Hyperion MicroReader™ 3 strip reader with 650 nm filter.
- (14) Timer.
- (15) Waterproof marker.
- (16) Microwell holder.

11.10 STORAGE CONDITIONS

- a. Store test kits between 36°- 46° F when not in use. Avoid prolonged storage of kits at room temperature. Do not freeze test kits.
- b. Do not use reagents from other SDI aflatoxin kits with different lot numbers.
- c. Bring kits up to room temperature 64° - 86° F prior to use.
- d. Do not use kit components beyond their expiration date.

1.5 APPROVED TEST METHODS

FGIS has approved test kits for use at field testing locations. The AflaCup, EZ-Screen, and Agri-Screen test kits are approved for qualitative analysis of corn. The Aflatest, Fluoroquant, Veratox-AST, and Myco✓ test kits provide quantitative analysis but can be used for qualitative results. HPLC testing is reserved for quantitative testing at TSD only.

The methods listed below have been conformance tested to perform within FGIS specifications. Each of the approved test methods has been certified to provide results accurate up to the conformance test level at which they were approved.

FGIS APPROVED TEST METHODS			
Method and Test Kit	Approved for		Conformance Limit(s)
	Qualitative	Quantitative	
AflaCup (International Diagnostics Inc.)	X		20 ppb
EZ-Screen - (Editek, Inc.)	X		20 ppb
AgriScreen - (Neogen)	X		20 ppb
Veratox AST - (Neogen)	X	X	300 ppb (quantitative)
Fluoroquant - (Romer)	X	X	300 ppb (quantitative)
Aflatest	X	X	300 ppb (quantitative)
Myco✓ - (Strategic Diagnostics Inc.)	X	X	300 ppb (quantitative)

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

GRAIN/ COMMODITY	TEST METHOD						
	AflaCup	Aflatest	EZ-Screen	Agri-Screen	Fluoroquant	Veratox-AST	Mycos [✓]
Corn	X	X	X	X	X	X	X
Sorghum		X			X	X	X
Wheat		X			X	X	
Soybeans		X			X	X	
Corn Screenings		(*)				(*)	
Corn Meal		X			X	X	X
Corn Germ Meal		X				X	
Corn Gluten Meal		X				X	
Corn/Soy Blend		X			X	X	X
Corn Gluten Feed		X					
Flaking Corn Grits		X			(*)	(*)	
Corn Flour						(*)	
Corn Bran						(*)	
Popcorn		X			X	X	X
Milled Rice		X			X	X	
Rough Rice						(*)	

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

The symbol (*) entered into a block denotes that the test kit is under evaluation by the Technical Services Division (TSD) for the grain/commodity and is temporarily approved for official use.

4.4 OPTIONAL STATEMENTS

a. Estimated Results.

At the request of the applicant, certify test results that exceed the conformance limit as "estimated" unless a supplemental analysis is performed.

"Aflatoxin (result) Estimated."

NOTE: Do not show "estimated" if solution was diluted and supplemental analysis performed.

b. Aflatoxin Not Detected.

At the request of the applicant, use the following statement when aflatoxin is not detected (0 ppb).

"Aflatoxin not detected."

NOTE: If subplot results are combined and averaged and the lot average is equal to 0 ppb, but an individual subplot result exceeds 0.0 ppb, the statement may not be used.

c. Converting to Parts per Million (ppm).

At the request of the applicant, convert and certify the ppb result to parts per million (ppm) using an approved statement. To convert ppb to ppm, divide the ppb result by 1000.

"(Actual ppb result) ppb is equivalent to (converted ppm results) ppm."

d. Converting to Milligrams (mg) per Kilogram (kg), or Micrograms (µg) per Kilogram (kg).

At the request of the applicant, convert and certify results in milligrams per kilogram (mg/kg) or micrograms per kilogram (µg/kg). Use the following equivalents to determine mg/Kg or µg /kg:

$$\text{ppm} = \text{mg/kg}$$

$$\text{ppb} = \mu\text{g /kg}$$

e. Multiple Results on the Same Certificate.

When certifying multiple aflatoxin results on the same certificate and the results are based on different sample types, the certificate must reflect the difference. As a guideline, the multiple results are shown as follows:

"Sublot sample results: Aflatoxin equal to or less than 20 ppb."

"Composite sample result: Aflatoxin 14 ppb."

f. Negative Result Statement.

At the request of the applicant, one of the following statements may precede the applicable standard statements when test results are equal to or less than 20 ppb.

"The aflatoxin result is negative." OR "Negative aflatoxin."

g. Type of Test Statement.

At the request of the applicant, use this statement to indicate the type of aflatoxin test used.

"Results based on (indicate type of test used) method."

NOTE: These certification statements may be modified as deemed necessary.

4.5 REVIEW INSPECTION STATEMENTS

Use the appropriate statements listed below for reinspection, appeal, and Board appeal inspections.

- a. Results are reported on the same kind of certificate issued for the original service and supersede the previously issued inspection certificate.

"This certificate supersedes Certificate No. (number) dated (date)."

- b. The superseded certificate is null and void as of the date of the subsequent (reinspection/appeal/Board appeal) certificate.

"The superseded certificate has not been surrendered."

CHAPTER 8

AFLATEST TEST METHOD

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8.1 GENERAL INFORMATION

The Aflatest method of testing for aflatoxin uses monoclonal antibody affinity chromatography that provides for quantitative measurement of total aflatoxins (B1, B2, G1, and G2) in parts per billion (ppb) or qualitative (screening) for aflatoxin.

8.2 PREPARATION OF SOLUTIONS

Prior to beginning test procedures, prepare the solutions required for testing. The distilled/deionized water, dilute developer solution, and the HPLC grade methanol must be checked for background fluorescence with the fluorometer after properly calibrated. None of the above reagents should give a positive reading of more than 1.0 ppb.

a. Dilute Developer Solution.

NOTE: Developer Solution must be prepared fresh daily.

The concentrated developer solution should have a slight reddish brown color. (Do not use the stock solution if it is colorless.) Loss of color indicates that the stock solution has lost its potency.

Prepare dilute developer solution by adding 5 ml of Aflatest developer concentrate (Vicam Cat. # 32010) to 45 ml of distilled/deionized water. Mix well, and label the dilute developer solution bottle showing the date and time of preparation.

DO NOT USE IT AFTER 6 HOURS HAVE ELAPSED.

If the amount of dilute developer being prepared needs to be adjusted based on the workload at individual locations, make sure that the 1 part concentrated developer to 9 parts distilled/deionized water ratio is maintained.

Label each stock bottle of concentrated developer with the date on which it was first opened. **DO NOT USE IT AFTER 30 DAYS HAVE ELAPSED.**

b. 80/20 Percent Methanol Solution.

Make up the solution by using the ratio of 8 parts HPLC grade methanol to 2 parts deionized/distilled water. Prepare the 80 percent methanol water solution by adding 800 ml methanol to 200 ml of water. Mix well. Keep the bottle tightly capped when not in use.

Label the 80 percent methanol/water solution bottle showing date of preparation. If the amount of the 80 percent methanol solution being prepared needs to be adjusted based on the workload at individual locations, make sure that the 8 parts HPLC grade methanol to 2 parts distilled/deionized water ratio is maintained.

To prepare smaller or larger amounts of solution the ratio of 8 parts methanol to 2 parts of deionized or distilled water must be maintained. For example: To prepare a solution that will provide for 5 test extractions (100 ml per test sample) mix 400 ml HPLC grade methanol to 100 ml deionized or distilled water.

8.3 FLUOROMETER CALIBRATION

a. General.

An FGIS-approved fluorometer is used to determine the aflatoxin level. To ensure accurate results, calibrate the fluorometer prior to use each day and verify at least once an hour using the **Yellow Vial**.

Turn the fluorometer on with the On/Off switch located on the rear panel. When the fluorometer is turned on, allow it to warm up for 10 minutes before calibrating. Once the fluorometer is turned on, it may be left on until close of business for the day. If the fluorometer is turned off during the day, a 10-minute warm up is required.

After turning the fluorometer on, it will identify itself and perform a set of self-tests. If any error message appears, consult the operator's manual.

b. Calibration Procedures.

- (1) Set the date, time, test delay time (60 seconds), and measurement units (ppb).
- (2) Follow the prompts on the fluorometer display to calibrate the unit.
- (3) When prompted to insert a calibration vial, wipe the vial with a clean cloth or paper wipe and insert it into the bottom of the well. Be sure that the vial is fully inserted and touches the bottom of the well.

- (4) Enter the correct calibration value (see table below) for the high calibrator (red vial) and low calibrator (green vial).

Note: This step is applicable to the Series III and Series IV fluorometers only. Calibration values are not entered for the MF-2000 Minifluorometer.

- (5) Check the calibration by testing the yellow vial.

Calibrations (in ppb) for Corn, Corn Meal, Corn/Soy Blend, Corn Germ Meal, Wheat, Sorghum, Soybeans, Flaking Corn Grits, Milled Rice, and Popcorn			
	<u>Series III</u>	<u>Series IV</u>	<u>MF-2000</u>
Red	150	140	*
Green	-3.0	-3.0	*
Yellow	75 ± 5	70 ± 5	66 - 74

Calibrations (in ppb) for Corn Gluten Meal and Corn Gluten Feed			
	<u>Series III</u>	<u>Series IV</u>	<u>MF-2000</u>
Red	110	100	*
Green	-3.0	-3.0	*
Yellow	55 ± 5	50 ± 5	66 - 74

*** Note: No values for the red and green calibrators.**

The MF-2000 does not give digital display values. Instead, a series of bar graph lights and the FGIS Aflatest overlay are used to read the yellow calibrator value. When the yellow vial is inserted, 10 bar graph lights should illuminate. This corresponds to a value between 66 - 74 ppb. Use the overlay to determine whether the value of the yellow vial is within FGIS specifications.

- (6) Record the result for the Yellow Vial.
- (7) If the value of the yellow calibration vial is not within FGIS specifications, repeat the calibration process (steps 2 through 4 listed above), then check the yellow vial again. If the reading for the Yellow Vial remains above or below FGIS specifications, contact the Mycotoxin Testing Group at TSD.
- (8) When the fluorometer is calibrated, place the standards back in the case and close tightly, and store away from any light source.
- (9) Check the calibration of the fluorometer at least once an hour or before analyzing any test samples if more than 1 hour time has elapsed since the last test using the Yellow Vial.

c. Calibration Standards.

(1) Maintenance.

The standard solutions in the three (3) standard vials (Red, Green, and Yellow) degrade slowly in the presence of light.

Since the plastic case containing the vials passes a small amount of light, it is recommended that both case and vials be stored in a cabinet or drawer away from all light except when calibrating or checking the calibration of the fluorometer.

Maintain two (2) sets of standards (two cases) at each location. Select and identify one set as the working standard, the other as the reference standard to be used to check the working standard every 14 days.

The degradation of the working set will occur gradually over a period of time, so anticipate expiration and requisition a replacement set in advance. (A sudden change in the reading of a vial indicates instrument instability, a cracked vial, or undue exposure of the vial to light.)

When one vial of a set expires, replace the entire set. About 2 months before the expected expiration of the working set, obtain a new set of standards from Vicam Co. When received, compare fluorometer readings of the new set with those of the existing reference set. If the difference between the two sets exceeds 3 ppb for any of the colors, notify TSD.

(2) Biweekly check of working standards.

Calibrate the fluorometer using the working set as described in "Calibration Procedures" (see section 8.3 b).

After calibrating the working set, remove the reference set from storage and test the 3 vials as described in section 8.4 b. The difference in readings of the two sets should not exceed the following limits:

<u>Red</u>	<u>Yellow</u>	<u>Green</u>
± 10 ppb	± 5 ppb	± 2 ppb

If the difference between the working and reference sets exceeds the tolerances, discard the working set. Begin using the old reference set as the working set, and use the new set as the reference set. Keep a permanent record of all calibration verification data.

8.4 SOLUTION TESTING

The distilled/deionized water, dilute developer solution, and HPLC grade methanol must be tested for background fluorescence before use. After calibrating the fluorometer perform the following:

a. Methanol.

Place 2.0 ml of HPLC grade methanol into a clean cuvette. Place the cuvette in the calibrated fluorometer. The displayed reading should be between -3.0 and +1.0. If the reading is positive and greater than 1.0, replace the methanol.

b. Water.

Dispense 2.0 ml of deionized/distilled water into a clean cuvette. Place the cuvette in the calibrated fluorometer. The digital display reading should be between -3.0 and +1.0. If the reading is positive and greater than 1.0, take action to assure a pure water supply.

c. Developer Solution.

Combine 1.0 ml of dilute developer solution and 1.0 ml of HPLC grade methanol in a clean cuvette. Place the cuvette in the calibrated fluorometer. The digital display reading should be between -3.0 and +1.0.

If the reading is positive and greater than 1.0, check each reagent separately to determine which reagent is causing the problem and replace it.

8.5 TEST PROCEDURES

a. Procedures for Testing Corn, Corn Meal, Corn/Soy Blend, Flaking Corn Grits, Milled Rice, Popcorn, Sorghum, and Soybeans.

Note: All aflatoxin tests for rice are performed on a milled rice basis. Consequently, rough rice or brown rice require milling before analysis. Mill rough rice or brown rice according to the procedures in the Rice Handbook.

(1) Extraction.

- (a) Place 50 g of ground sample into blender jar.
- (b) Add 5 grams of analytical, USP grade sodium chloride (NaCl) or food grade un-iodized salt.
- (c) Add 100 ml of the 80/20 methanol/water extraction solution.
- (d) Cover jar and blend at high speed for 1 minute.
- (e) Remove the cover and pour the extract into a filter paper (Whatman 2V folded or S&S 591 24 cm pleated or equivalent) supported in a clean funnel.
- (f) Collect the filtrate in a clean beaker labeled with the sample identification.
- (g) After collecting approximately 25 ml of extract, carefully dispose of the filter paper and its contents.
- (h) Pipette 5 ml of filtered extract into a clean beaker.

- (i) Add 10 ml of distilled/deionized water and mix thoroughly.
- (j) Filter the diluted extract through a glass microfibre filter (Vicam Cat. # 31955) supported by a small, clean funnel. Fold the glass microfibre filter gently without making a sharp crease to avoid breaking the glass microfibre filter.
- (k) Immediately proceed with the Aflatest Affinity Column procedure.

Note: If this diluted filtrate turns cloudy, refilter using a new glass microfibre filter before proceeding with the analysis.

(2) Affinity Column.

- (a) Prepare an Aflatest-P affinity column for use by removing both end caps and gently shaking the buffer solution from the top of the column.
- (b) Using an Eppendorf pipette, add 1.0 ml of the filtered dilute extract to the top of the Aflatest column.
- (c) Attach the column to the washing device (either a syringe barrel or an air pumping station) and pass the filtered extract through the column using a steady positive pressure. Maintain a flow rate of approximately 1 drop per second.

Note: Sample analysis using these procedures can be greatly simplified by the use of a small aquarium air pump to provide the needed air pressures for loading, filtering, and washing the various extracts.

- (d) After the extract has completely passed through the Aflatest column, add 1 ml of deionized or distilled water to the column and again apply a steady positive pressure to pass the wash water through the column. (If a syringe barrel rather than the pumping station is used, detach the column and pipette 1 ml of deionized or distilled water into the column headspace.) Reattach the column to the syringe barrel and apply pressure to pass the water through the column.

- (e) Repeat the water wash in step (d) above.
 - (f) After the second wash has passed through the column, place a clean cuvette under the outlet of the column. Only 12 x 75 mm borosilicate glass tubes should be used for cuvettes (Vicam Cat. # 34000 or equivalent). Use care when handling the cuvette to keep the optical surface clean and free of lint, fingerprints, etc.
 - (g) Dispense 1.0 ml of HPLC grade methanol into the column. If a syringe barrel rather than the pumping station is used, detach the column, pipette 1 ml of methanol directly into the column headspace, and replace the column.
 - (h) Apply a steady pressure to elute/pass the methanol through the column and collect all of the methanol eluate in the cuvette. Maintain pressure to collect the methanol at a rate of approximately 1 drop per second.
 - (i) Add 1.0 ml of dilute Aflatest Developer Solution directly to the sample eluate solution in the cuvette and mix well (about 5 seconds).
 - (j) **Immediately** place the cuvette in a calibrated fluorometer.
- (3) Reading, Recording, and Certifying Test Results.
- (a) Record the digital readout (Series III and IV) or corresponding bar graph value (MF-2000) as total ppb.
 - (b) Report all results on the pan ticket and the inspection log to the nearest whole ppb.
 - (c) Sample results over 300 ppb are reported as >300 ppb unless a supplemental analysis is performed.
 - (d) Refer to the Certification section of the handbook for more detailed certification procedures.

(4) Supplemental Analysis.

To determine and report an aflatoxin level higher than 300 ppb, the filtered test sample extract must be diluted so that a value between 5 ppb and 300 ppb is obtained. The final aflatoxin concentration is calculated by multiplying the results obtained with the diluted extract by the dilution factor.

- (a) Using an Eppendorf pipette, add 0.5 ml (instead of 1.0 ml) of the filtered diluted extract to the top of the Aflatest column headspace. (See section 8.5 a (2) (b).)
- (b) Analyze the filtered extract as a normal sample.
- (c) Multiply the analytical results obtained by 2 to obtain the actual aflatoxin concentration. For example, if 240 ppb was the sample value obtained using the diluted test sample procedure, the actual concentration in the original sample was 480 ppb.

Example:	Diluted test sample extract result	240 ppb
	Dilution factor	<u>x 2</u>
	Actual aflatoxin concentration	480 ppb

Note: Laboratories may dilute samples as a first step if levels typically observed exceed 300 ppb and the applicant requests actual (not estimated) readings.

b. Procedures for Testing Corn Germ Meal and Wheat.

(1) Extraction.

- (a) Place 50 g of ground sample into blender jar.
- (b) Add 10 grams of analytical, USP grade sodium chloride (NaCl) or food grade un-iodized salt.
- (c) Add 200 ml of the 80/20 methanol/water extraction solution.
- (d) Cover jar and blend at high speed for 1 minute.

- (e) Remove the cover and pour the extract into a filter paper (Whatman 2V folded or S&S 591 24 cm pleated or equivalent) supported in a clean funnel.
- (f) Collect the filtrate in a clean beaker labeled with the sample identification.
- (g) After collecting approximately 25 ml of extract, carefully dispose of the filter paper and its contents.
- (h) Pipette 5 ml of filtered extract into a clean beaker.

Note: If the solution filtration is slow (i.e., more than two minutes are required to collect 5 ml of filtrate), withdraw 5.0 ml of the clearest liquid from the top of the material held in the funnel (see step (e) above) and transfer it to a clean container.

- (i) Add 10 ml of distilled/deionized water and mix thoroughly.
- (j) Filter the diluted extract through a glass microfibre filter (Vicom Cat. # 31955) supported by a small, clean funnel. Fold the glass microfibre filter gently without making a sharp crease to avoid breaking the glass microfibre filter.
- (k) Immediately proceed with the Aflatest Affinity Column procedure.

Note: If this diluted filtrate turns cloudy, refilter using a new glass microfibre filter before proceeding with the analysis.

(2) Affinity Column.

- (a) Prepare an Aflatest-P affinity column for use by removing both end caps and gently shaking the buffer solution from the top of the column.
- (b) Using an Eppendorf pipette, add 1.0 ml of the filtered dilute extract to the top of the Aflatest column.

- (c) Attach the column to the washing device (either a syringe barrel or an air pumping station) and pass the filtered extract through the column using a steady positive pressure. Maintain a flow rate of approximately 1 drop per second.

Note: Sample analysis using these procedures can be greatly simplified by the use of a small aquarium air pump to provide the needed air pressures for loading, filtering, and washing the various extracts.

- (d) Using an Eppendorf pipette, add 1.0 ml of the filtered dilute extract to the top of the Aflatest column.
- (e) Attach the column to the washing device (either a syringe barrel or an air pumping station) and pass the filtered extract through the column using a steady positive pressure. Maintain a flow rate of approximately 1 drop per second.
- (f) After the extract has completely passed through the Aflatest column, add 1 ml of deionized or distilled water to the column and again apply a steady positive pressure to pass the wash water through the column. (If a syringe barrel rather than the pumping station is used, detach the column and pipette 1 ml of deionized or distilled water into the column headspace.) Reattach the column to the syringe barrel and apply pressure to pass the water through the column.
- (g) Repeat the water wash in step (f) listed above.
- (h) After the second wash has passed through the column, place a clean cuvette under the outlet of the column. Only 12 x 75 mm borosilicate glass tubes should be used for cuvettes (Vicom Cat. # 34000 or equivalent). Use care when handling the cuvette to keep the optical surface clean and free of lint, fingerprints, etc.
- (i) Dispense 1.0 ml of HPLC grade methanol into the column. If a syringe barrel rather than the pumping station is used, detach the column, pipette 1 ml of methanol directly into the column headspace, and replace the column.

- (j) Apply a steady pressure to elute/pass the methanol through the column and collect all of the methanol eluate in the cuvette. Maintain pressure to collect the methanol at a rate of approximately 1 drop per second.
- (k) Add 1.0 ml of dilute Aflatest Developer Solution directly to the sample eluate solution in the cuvette and mix well (about 5 seconds).
- (l) **Immediately** place the cuvette in a calibrated fluorometer.

(3) Reading, Recording, and Certifying Test Results.

- (a) Record the digital readout (Series III and IV) or corresponding bar graph value (MF-2000) as total ppb.
- (b) Report all results on the pan ticket and the inspection log to the nearest whole ppb.
- (c) Sample results over 300 ppb are reported as >300 ppb unless a supplemental analysis is performed.
- (d) Refer to the Certification section of the handbook for more detailed certification procedures.

c. Procedures for Testing Corn Gluten Meal and Corn Gluten Feed.

(1) Extraction.

- (a) Place 50 g of ground sample into blender jar.
- (b) Add 5 grams of analytical, USP grade sodium chloride (NaCl) or food grade un-iodized salt.
- (c) Add 250 ml of 60 percent methanol/40 percent water extraction solution to blender jar. The 60/40 percent methanol/water solution is prepared by mixing 600 ml HPLC grade methanol with 400 ml distilled/deionized water.
- (d) Cover jar and blend at high speed for 1 minute.

- (e) Remove the cover and pour the extract into a filter paper (Whatman 2V folded or S&S 591 24 cm pleated or equivalent) supported in a clean funnel.
 - (f) Collect the filtrate in a clean beaker labeled with the sample identification.
 - (g) After collecting approximately 25 ml of extract, carefully dispose of the filter paper and its contents.
 - (h) Pipette 10 ml of filtered extract into a clean beaker.
 - (i) Add 20 ml of distilled/deionized water and mix thoroughly.
 - (j) Filter the diluted extract through a glass microfibre filter (Vicom Cat. # 31955) supported by a small, clean funnel. Fold the glass microfibre filter gently without making a sharp crease to avoid breaking the glass microfibre filter.
 - (k) Load 6-8 ml of the filtrate from step (j) above into a 10 ml plastic syringe barrel fitted with 0.22 micron nylon syringe disk filter (Fisher Scientific Corporation CAMEO II Cat. No. DDN 02T2550, Gelman Cat. No. 09-730-191, or Corning Cat. No. 09-754-22).
 - (l) Apply enough air pressure to syringe barrel to produce a flow of approximately 1 drop per second through disk filter and collect a minimum of 5 ml of filtrate in a clean test tube. Discard filter disk.
- (2) Affinity Column.
- (a) Prepare an Aflatest-P affinity column for use by removing both end caps and gently shaking the buffer solution from the top of the column.
 - (b) Using a 1.0 ml Eppendorf pipette, load 4.0 ml of refiltered extract from step (l) above into the barrel of a 10 ml glass syringe to which an Aflatest-P column is attached.

- (c) Apply pressure so that the extract passes through the column at 1 to 2 drops per second. Remove syringe barrel from column. Fill column with distilled water. Reattach syringe barrel to column.
- (d) Fill syringe barrel with 10 ml of distilled/deionized water and pass through column at a flow rate of approximately 2 drops per second. Allow all of wash water to pass through column.
- (e) Repeat column wash with another 10 ml of deionized/distilled water.
- (f) Elute aflatoxin from Aflatest-P column with 1 ml HPLC grade methanol and collect sample eluate solution in glass cuvette.
- (g) Add 1 ml of fresh, dilute Aflatest developer solution directly to the eluate in cuvette and mix well.
- (h) **Immediately** place the cuvette in a calibrated fluorometer.

(3) Reading, Recording and Certifying Test Results.

- (a) Record the digital readout (Series III and IV) as total ppb. **To determine the aflatoxin concentration using the MF-2000 fluorometer, read the corresponding bar graph value and multiply by 0.73 for actual ppb.**
- (b) Report all results on the pan ticket and the inspection log to the nearest whole ppb.
- (c) Sample results over 300 ppb are reported as >300 ppb.
- (d) Refer to the Certification section of the handbook for more detailed certification procedures.

NOTE: Rinse both glass and plastic syringe barrels with approximately 10 ml of distilled/deionized water each before analyzing next sample.

8.6 CLEANING LABWARE

a. Negative Tests (# 20 ppb).

(1) 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.

(2) Disposable Materials.

Place materials in a garbage bag for routine trash disposal.

b. Positive Tests (> 20 ppb).

(1) Labware.

Prepare a bleach solution consisting of 1 part bleach to 10 parts water (e.g., 100 ml bleach to 1,000 ml water). Completely submerge the used glassware, funnels, beakers, etc., and soak for at least 5 minutes. Remove items from the bleach/water solution, submerge in a dishwashing liquid/water solution, wash thoroughly, then rinse with clean water before reusing.

(2) Disposable Materials.

Prepare a bleach solution consisting of 1 part bleach to 10 parts water in a plastic pail labeled "bleach solution". Soak disposable materials, such as used columns, cuvettes, vials, test kit components, etc., for at least 5 minutes. Pour off the liquid down the drain and place the materials in a garbage bag and discard.

8.7 WASTE DISPOSAL

a. Negative Results (# 20 ppb).

If the test result is negative (equal to or less than 20 ppb), discard the filter paper and its contents (ground material) into a plastic garbage bag for disposal. Dispose of any remaining liquid filtrate in the chemical waste container.

b. Positive Results (> 20 ppb).

If the result is positive (more than 20 ppb), the ground portion remaining in the filter paper must be decontaminated prior to disposal. After disposing of the remaining filtered extract in the chemical waste container, filter approximately 50 ml of bleach through the filter containing the ground portion and allow to drain. Discard the filter paper and its contents (ground portion) into a plastic garbage bag for disposal. The bleach rinse filtrate collected may be treated as a non-hazardous solution and disposed of by pouring down the drain.

8.8 EQUIPMENT and SUPPLIES

- a. Fluorometer - Romer model RL-100, Vicam Series III and IV, or Vicam model MF-2000.
- b. Fluorometer calibration standards. (Vicam # 33050)
- c. Cuvette Rack. (Vicam # 21010)
- d. Pump assembly stand, double. (Vicam # 21030)
- e. Syringe, glass 10 ml. (Vicam # 34010)
- f. Syringe hand pump with coupling. (Vicam #36030)
- g. Automatic pipettor (1 ml capacity for methanol). (Vicam #20501)
- h. Automatic pipettor (1 ml capacity for developer). (Vicam #20600)
- i. Graduated cylinders - 25 ml, 100 ml, and 250 ml capacity.
- j. Aflatest-P columns. (Vicam # 12022)
- k. Cuvettes, disposable 12 x 75 mm borosilicate glass tube. (Vicam # 34000)
- l. Disposable beakers. (Vicam # 36010)
- m. Glass microfibre filter paper -Whatman 934-AH. (Vicam # 31955)
- n. Small plastic funnels.

- o. Wash bottles or spray bottles.
- p. Box of Kim Wipes (small size sheets).
- q. HPLC grade methanol.
- r. Aflatest developer solution. (Vicam # 32010)
- s. Balance.
- t. Sample Grinder.
- u. Distilled/deionized water.
- v. Aflatest developer solution. (Vicam #32010)
- w. USP grade sodium chloride (NaCl) or food grade un-iodized salt.

8.9 STORAGE CONDITIONS

- a. Affinity Columns - Store at room temperature (64° to 86° F).
- b. Calibration Vials - Store in a cabinet or drawer away from all light, except when in use.
- c. Developer Concentrate - Store in a tightly closed bottle in a cool, dry, well ventilated area and away from sunlight, combustible materials, and incompatible materials.

- (3) Place the blue cap on top of the column and mix thoroughly by hand, shaking vigorously for 5 seconds.
- (4) Uncap the top and bottom of the column and place the column in a 12 x 75 mm cuvette. Insert the syringe barrel and stopper into the top of the column.
- (5) Slowly (**30-40 seconds**) push the extract through the column until air comes out of the bottom.

NOTE: It is critical to push the solution completely through the column in at least a 30-40 second time-frame indicated to insure a complete extraction solution purification.

- (6) Transfer 250 μ l (0.25 ml) of each purified sample extract to a clean 12 x 75 mm cuvette.
- (7) Add 250 μ l of methanol to the cuvette and cap.

c. Derivatization and Fluorometric Reading.

- (1) Immediately add 1 ml of the developer working reagent to each purified sample.
- (2) Recap the tube and vortex for 5 seconds.
- (3) Wipe the cuvette with lint-free paper and place in the fluorometer for a reading.
- (4) After a 40-second delay, the result will appear on the fluorometer screen and a record will be printed out.

NOTE: Once the developer reagent is added, the sample must be mixed, and the sample cuvette must be placed in the fluorometer quickly. Samples must be derivatized one sample at a time and then read before proceeding to the next sample.

9.6 REPORTING AND CERTIFYING TEST RESULTS

- a. Record the digital readout as ppb total aflatoxins in the sample.
- b. Report all results on the pan ticket and the inspection log to the nearest whole ppb.
- c. Sample results over 300 ppb are reported as >300 ppb unless a supplemental analysis is performed.
- d. Refer to the Certification section of the handbook for more detailed certification procedures.

9.7 SYSTEM CHECK

a. Positive Control Option

The test kit contains a positive control standard that may be used as a check on method technique and overall system performance.

b. System Check Procedures

- (1) Make sure the clear plastic tip is pushed firmly onto the bottom of the UniSep2001 column.
- (2) Place 1 ml of **80/20 methanol/water solution** in the top of the column and discard the pipette tip.

Note: Do not use the 90/10 acetonitrile/water solution in this procedure.

- (3) Add 1 ml of Positive Control Standard and discard the pipette tip.
- (4) Place the blue cap on top of the column and mix thoroughly by hand shaking vigorously for 5 seconds.
- (5) Uncap the top and bottom of the column and place the column in a 12 x 75 mm cuvette. Insert the syringe barrel and stopper into the top of the column.
- (6) Slowly (**30-40 seconds**) push the extract through the column until air comes out the bottom.

CHAPTER 5

AFLACUP TEST KIT

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5.1 GENERAL INFORMATION

The AflaCup test kit is an enzyme linked immunosorbent assay (ELISA) developed by International Diagnostics Systems Corporation and marketed by Romer Labs. As the name implies, antibodies which react specifically with aflatoxins are contained in a cup. The test provides qualitative (less than or equal to a specified threshold) results.

5.2 PREPARATION OF EXTRACTION SOLUTION

The extraction solvent used in the AflaCup test method is a methanol/water (distilled or deionized) mixture consisting of 80 percent methanol (Reagent grade or better) and 20 percent water.

- a. Using a graduated cylinder, measure 800 ml of methanol and place it into a clean carboy with spigot.
- b. Add 200 ml deionized or distilled water to the methanol and shake vigorously until it is completely mixed.
- c. Label the container stating the mixture (80 percent methanol and 20 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 8 parts methanol to 2 parts of deionized or distilled water.

5.3 EXTRACTION PROCEDURES

- a. Transfer 50 grams of ground sample into an extraction mixing jar.
- b. Add 100 ml of the (80/20) methanol/water extraction solvent.
- c. Cover the extraction jar and blend on high speed for 1 minute.
- d. Remove the cover and funnel the extract through a Whatman No.1 filter or a coffee filter into a sample jar labeled with the sample identification.
- e. After collecting the filtrate, remove the funnel, filter, and ground material and place over an empty collection container.

5.4 REAGENT CHECK

a. Stabilization.

Prior to performing the test, allow one hour for all reagents to reach room temperature (73E- 84EF).

b. Testing Reagents.

Each day, before testing official samples, test at least one negative control cup to ensure that all reagents are functional.

Use the following procedures to test the control:

- (1) Apply 2 drops of negative control (green cap) to the center of the AflaCup.
- (2) Using a timer, allow the cup to set for a 1-minute reaction time.
- (3) Apply 2 drops of the aflatoxin enzyme (red cap) to the center of the cup.

Note: The enzyme solution may only be used with the antibody-coated cups contained in the same test kit.

- (4) Using a timer, allow the cup to set for a 1-minute reaction time.
- (5) Wash with 30 drops of the Wash Solution (white cap). When using more than one AflaCup, wash each cup with 3 series of 10 drops per cup.
- (6) Prepare fresh Substrate Solution in a small test tube by mixing 10 drops of Substrate Solution A (yellow cap) with 10 drops of Substrate Solution B (blue cap) for each AflaCup.

(Do not combine Substrate Solution A with Substrate Solution B more than 10 minutes before use.)

Note: If a blue color develops immediately after combining Substrates A and B, repeat this step. If the problem persists call Romer Labs for technical assistance.

- (7) Add the entire contents of the Substrate Mix from each test tube to each test cup in use.
- (8) Using a timer, allow the cup to set for a 1-minute reaction time.
- (9) Immediately read and interpret the result.

c. Interpreting Results.

A blue color indicates the reagents are functional. If the color remains white for at least one minute the reagents are not functional and must be replaced.

5.5 TEST PROCEDURES

a. Procedures for a 20 ppb cut-off.

- (1) Transfer 200 microliters (μl) of the dilution buffer to a culture tube (12x 75 mm) and then add 100 μl of the filtered extract.
- (2) Mix well and slowly apply 100 μl of the mixture to the center of the AflaCup.
- (3) Using a timer, allow the cup to set for a 1-minute reaction time.
- (4) Apply 2 drops of the aflatoxin enzyme (red cap) to the center of the cup.

Note: The enzyme solution may only be used with the antibody-coated cups contained in the same test kit.

- (5) Using a timer, allow the cup to set for a 1-minute reaction time.
- (6) Wash with 30 drops of the Wash Solution (white cap). When using more than one AflaCup, wash each cup with 3 series of 10 drops per cup.
- (7) Prepare fresh Substrate Solution in a small test tube by mixing 10 drops of Substrate Solution A (yellow cap) with 10 drops of Substrate Solution B (blue cap) for each AflaCup.

(Do not combine Substrate Solution A with Substrate Solution B more than 10 minutes before use.)

Note: If a blue color develops immediately after combining Substrates A and B, repeat this step. If the problem persists call Romer Labs for technical assistance.

(8) Add the entire contents of the Substrate Mix from each test tube to each test cup in use.

(9) Using a timer, allow the cup to set for a 1-minute reaction time.

b. Interpretation of Test Results.

(1) Negative (equal to or less than 20 ppb).

The sample is considered equal to or less than 20 ppb when the cup color changes to blue.

Note: Color will be concentrated in the center of the cup.

(2) Positive (Greater than 20 ppb).

The sample is considered greater than 20 ppb when the cup color remains white for at least one minute.

5.6 REPORTING AND CERTIFYING TEST RESULTS

- a. Report results on the pan ticket and inspection log as being equal to or less than a threshold (e.g., 20 ppb) or as exceeding the threshold.
- b. Certify results as being equal to or less than a threshold.
- c. Refer to the Certification section of the handbook for more detailed certification procedures.

5.7 CLEANING LABWARE

a. Negative Tests (# 20 ppb).

(1) 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.

(2) Disposable Materials.

Place materials in a garbage bag for routine trash disposal.

b. Positive Tests (> 20 ppb).

(1) Labware.

Prepare a bleach solution consisting of 1 part bleach to 10 parts water (e.g., 100 ml bleach to 1,000 ml water). Completely submerge the used glassware, funnels, beakers, etc., and soak for at least 5 minutes. Remove items from the bleach/water solution, submerge in a dishwashing liquid/water solution, wash thoroughly, then rinse with clean water before reusing.

(2) Disposable Materials.

Prepare a bleach solution consisting of 1 part bleach to 10 parts water in a plastic pail labeled "bleach solution". Soak disposable materials, such as used columns, cuvettes, vials, test kit components, etc., for at least 5 minutes. Pour off the liquid down the drain and place the materials in a garbage bag and discard.

5.8 WASTE DISPOSAL

a. Negative Results (# 20 ppb).

If the test result is negative (equal to or less than 20 ppb), discard the filter paper and its contents (ground material) into a plastic garbage bag for disposal. Dispose of any remaining liquid filtrate in the chemical waste container.

b. Positive Results (> 20 ppb).

If the result is positive (more than 20 ppb), the ground portion remaining in the filter paper must be decontaminated prior to disposal. After disposing of the remaining filtered extract in the chemical waste container, filter approximately 50 ml of bleach through the filter containing the ground portion and allow to drain. Discard the filter paper and its contents (ground portion) into a plastic garbage bag for disposal. The bleach rinse filtrate collected may be treated as a non-hazardous solution and disposed of by pouring down the drain.

5.9 EQUIPMENT AND SUPPLIES

a. Materials Supplied in Test Kits:

- (1) Cups with Aflatoxin Antibody attached.
- (2) Aflatoxin Enzyme, Dropper Bottle.
- (3) Negative Control Solution, Dropper Bottle.
- (4) Wash Solution, Dropper Bottle.
- (5) Substrate A, Dropper Bottle.
- (6) Substrate B, Dropper Bottle.
- (7) Dilution Buffer for Samples, Translucent.

b. Materials Required but not Provided:

- (1) Sample grinder.
- (2) Balance.

- (3) Methanol - Reagent grade or better.
- (4) Distilled or deionized water.
- (5) Blender with mixing jars.
- (6) Cuvette rack.
- (7) Pipettor and tips - 100 to 1000 μ l adjustable.
- (8) 100 ml graduated cylinder.
- (9) Funnel.
- (10) Timer.
- (11) Whatman No.1 Filter Paper or Coffee Filters.
- (12) Glass cuvettes (12 x 75 mm).

5.10 STORAGE CONDITIONS

Test kits should be refrigerated between 36E- 46EF.

3.1 GENERAL INFORMATION

The manner in which samples are obtained and processed is an important consideration when testing for aflatoxin. To ensure that the test results accurately reflect the aflatoxin concentration present in a lot, samples must be representative of the lot and of sufficient size to compensate for the uneven distribution of the contaminant.

3.2 SAMPLE SIZE

Obtain samples according to the instruction 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 (lbs.) / grams
Trucks	2 pounds / approximately 908 grams
Railcars	3 pounds / approximately 1,362 grams
Barges/Sublots	10 pounds / approximately 4,540 grams

NOTE: A 10-pound sample size is also recommended, but not required, for submitted samples.

3.3 WORK RECORDS

Each testing laboratory must maintain work records for each test that include the name of the applicant, date of service, sample or carrier identification, test results, initials of official personnel performing the test, and any other information deemed necessary to properly certificate the test results and bill the applicant. As practical, use existing forms, such as FGIS-992, "Services Performed Report;" FGIS-920, "Grain Sample Ticket;" or FGIS-921, "Inspection Log," to record laboratory results.

Any sample sent to TSD (including the Board of Appeals and Review) for aflatoxin testing or monitoring purposes must include the necessary information to facilitate sample processing and testing.

3.4 SAMPLE PORTIONS

a. Subportions.

Grind the entire sample obtained for aflatoxin testing and prepare two 500-gram subportions from the ground sample: 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.

For subplot testing of corn at export locations, save an additional 500-gram file (three 500-gram subportions total) for Food and Drug Administration (FDA) analysis.

From the 500-gram work portion, divide (using a Boerner divider) out a portion of 50 grams for aflatoxin testing and weigh on an FGIS-approved type scale with a minimum division size of 0.1 gram.

b. Saving File Samples.

Maintain file samples (including the FDA file sample when applicable) for all lots/samples that:

do not meet the contractual specification of the applicant for service;

are required for the aflatoxin monitoring program; or

exceed FDA action limits of 20 ppb.

When applicable, maintain a representative file sample for each lot, subplot, 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 representativeness of the sample and prevent possible 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.)

U.S. DEPARTMENT OF AGRICULTURE
GRAIN INSPECTION, PACKERS AND STOCKYARDS
ADMINISTRATION
FEDERAL GRAIN INSPECTION SERVICE
STOP 3630
WASHINGTON, D.C. 20090-3630

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