FOOD CHEMICAL CONTAMINANTS

Validation Study of a Rapid ELISA for Detection of Aflatoxin in Corn

***Performance Tested Method*[SM] 050901**

Abstract

Neogen Corp. developed the Veratox aflatoxin test kit for the detection of total aflatoxin. The purpose of this study was to validate the method under the requirements of the AOAC Research Institute *Performance Tested Methods*[SM] (PTM) program. There are several AOAC *Official Methods*[SM] for total aflatoxin detection in corn (**994.08**, **990.33**, **979.18**, **993.17**, **990.32**, **993.16**, **991.31**, and **990.74**), varying between rapid and analytical-based methods and one rapid method that has been performance tested by the AOAC Research Institute (PTM 030701). However, the widely used reference method is AOAC *Official Method*[SM] **994.08**, which is an HPLC method and is referred to as the reference method in this paper. Although considered the reference method, the HPLC procedure is complicated and requires the investment of both expensive equipment and a highly skilled technician. A rapid (e.g., ELISA) test kit to be validated by the AOAC Research Institute is needed.

1 Scope of Method

1.1 Target Analyte

The mycotoxin aflatoxin (total = B_1, B_2, G_1, and G_2).

1.2 Matrix

Corn.

1.3 Summary of Validated Performance Claims

Based on internal validation study:

Precision.—Less than 21% CV average of all levels tested.

Accuracy.—94.85% agreement with HPLC with >95% aflatoxin recovery observed.

Cross-reactivity with closely related compound.—None.

LOD.—1.4 ppb.

Range of quantitation.—This assay has a range of quantitation between 5.0 and 50 ppb without additional dilution, and range can be extended through further dilution with 70% methanol.

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The method was independently tested, evaluated, and certified by the AOAC Research Institute as a *Performance Tested Method*[SM]. *See* http://www.aoac.org/testkits/steps.html for information on certification.

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1.4 Definitions

Precision.—The %CV of multiple assays of the same sample, where %CV = (SD/mean 100%; 1).

Accuracy.—Agreement with reference method value.

LOD.—The mean value of 10 negative samples plus two SD values.

Range of quantitation.—Range at which the results obtained can be accurate.

Detection limit.—The minimum amount of analyte required to elicit a positive result, which is understood to be greater than the values listed for LOD above.

2 Participants

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3 Introduction

Aflatoxins are widely considered one of the most potent naturally occurring carcinogens. Currently, the FDA regulates the amount of aflatoxins allowed in human foods and animal feeds to 20 g/kg. There are currently several methods for quantitatively detecting total aflatoxins that are AOAC *Official Methods*[SM] (2–4); however, none are quantitative ELISA methods. There are also various

Table 1. HPLC determinations on naturally contaminated corn samples[a]

	5 ppb					10 ppb					20 ppb					100 ppb				
	AflaB1	AflaB2	AflaG1	AflaG2	Total	AflaB1	AflaB2	AflaG1	AflaG2	Total	AflaB1	AflaB2	AflaG1	AflaG2	Total	AflaB1	AflaB2	AflaG1	AflaG2	Total
									Run 1											
1	5.5	0.4	ND	ND	5.9	7.9	0.4	ND	ND	8.3	16.5	1.2	ND	ND	17.7	104	8	ND	ND	112
2	5.5	0.4	ND	ND	5.9	10.3	0.5	ND	ND	10.8	17.9	1.2	ND	ND	19.1	112.8	8.3	ND	ND	121.1
3	5	0.2	ND	ND	5.2	8.2	0.5	ND	ND	8.7	17.6	1.1	ND	ND	18.7	94.5	7.7	ND	ND	102.2
4	5.6	0.3	ND	ND	5.9	9.2	0.5	ND	ND	9.7	18.3	1.2	ND	ND	19.5	115.9	9.1	ND	ND	125
5	5.6	0.4	ND	ND	6.0	9.2	0.6	ND	ND	9.8	15.1	0.7	ND	ND	15.8	98.5	7.4	ND	ND	105.9
6	4.6	0.3	ND	ND	4.9	8.7	0.3	ND	ND	9.0	17.7	0.9	ND	ND	18.6	96.5	7.5	ND	ND	104
7	5.4	0.3	ND	ND	5.7	9.4	0.4	ND	ND	9.8	17.9	0.8	ND	ND	18.7	95.6	7.1	ND	ND	102.7
									Run 2											
1	5	0.3	ND	ND	5.3	10.4	0.3	ND	ND	10.7	17.4	1.1	ND	ND	18.5	98.1	6.7	ND	ND	104.8
2	4.4	0.3	ND	ND	4.7	9.6	0.3	ND	ND	9.9	15.8	0.7	ND	ND	16.5	94.6	6.5	ND	ND	101.1
3	6	0.3	ND	ND	6.3	7.5	0.5	ND	ND	8.0	16.9	1.2	ND	ND	18.1	115.6	8.4	ND	ND	124
4	5.9	0.3	ND	ND	6.2	7.9	0.4	ND	ND	8.3	17.3	1.1	ND	ND	18.4	95.4	6.3	ND	ND	107.7
5	6.2	0.3	ND	ND	6.5	6.6	0.3	ND	ND	6.9	15.7	0.9	ND	ND	16.6	110.8	7.5	ND	ND	118.3
6	4.7	0.3	ND	ND	5.0	7.8	0.4	ND	ND	8.2	17.6	0.8	ND	ND	18.4	107.7	7.3	ND	ND	115
7	5.9	0.3	ND	ND	6.2	9.6	0.4	ND	ND	10.0	18.6	1.2	ND	ND	19.8	106.8	7.7	ND	ND	114.5
									Run 3											
1	5.3	0.3	ND	ND	5.6	7.2	0.5	ND	ND	7.7	16.4	1.2	ND	ND	17.6	97.3	7.4	ND	ND	104.7
2	5.1	0.4	ND	ND	5.5	8.8	0.7	ND	ND	9.5	15.5	0.9	ND	ND	16.4	109.1	8.4	ND	ND	117.5
3	6.1	0.3	ND	ND	6.4	8.5	0.6	ND	ND	9.1	18.2	1.2	ND	ND	19.4	107.4	9.2	ND	ND	116.6
4	5.9	0.3	ND	ND	6.2	10.8	0.8	ND	ND	11.6	16.6	1.2	ND	ND	17.8	106.3	8	ND	ND	114.3
5	5.2	0.3	ND	ND	5.5	9.5	0.5	ND	ND	10.0	15.8	0.7	ND	ND	16.5	97.2	7.6	ND	ND	104.8
6	5.2	0.3	ND	ND	5.5	8.6	0.7	ND	ND	9.3	17.6	0.8	ND	ND	18.4	111.5	8.4	ND	ND	119.9
7	5	0.3	ND	ND	5.3	8	0.6	ND	ND	8.6	15.7	0.9	ND	ND	16.6	109.3	8.5	ND	ND	117.8
Mean					5.7					9.2					18					111.8
SD					0.5					1.1					1.2					8
CV					9.0					12.3					6.4					7.1

[a] All results in ppb.

Table 2A. Accuracy and precision of Veratox aflatoxin ELISA[a]

	Analyst											
	5.7 ppb			9.2 ppb			18.0 ppb			111.8ppb		
	1	2	3	1	2	3	1	2	3	1	2	3
	5.70	6.40	6.30	11.60	11.40	12.30	22.30	20.80	19.80	105.20	100.00	94.00
	6.20	6.20	6.10	9.70	11.60	10.90	20.60	24.10	20.30	107.20	103.20	102.00
	6.30	5.60	6.00	10.20	9.90	11.20	19.00	19.80	19.00	101.20	102.00	94.80
	7.20	6.10	6.70	9.50	10.80	11.10	22.50	18.80	20.00	109.20	92.40	94.40
	5.90	7.20	7.00	9.10	11.00	11.50	23.60	18.50	22.20	106.40	98.00	94.00
	5.80	7.20	9.00	10.30	11.00	11.00	24.80	19.20	21.20	104.00	97.60	89.60
	6.50	6.00	7.30	8.10	9.50	10.50	23.40	16.80	19.60	98.00	76.40	87.20
	7.00	6.30	5.40	10.30	13.60	12.50	18.10	18.00	18.60	94.80	93.60	82.40
	6.60	6.00	5.60	8.40	11.20	9.60	20.10	19.80	19.80	91.20	94.80	87.20
	5.50	5.80	4.60	11.60	14.20	12.00	19.40	20.60	20.30	98.40	99.60	84.80
	6.20	7.00	5.50	8.00	10.40	9.60	18.60	19.00	20.80	98.40	95.20	88.00
	6.00	6.40	5.20	6.90	10.20	10.30	19.80	21.20	22.00	98.40	105.60	101.60
	7.10	8.60	5.70	6.80	10.80	10.80	20.10	19.80	21.30	91.20	92.40	87.20
	5.70	6.50	5.90	8.60	9.80	10.30	16.20	17.30	17.50	82.00	76.40	85.20
	4.80	6.10	5.20	9.70	8.40	9.60	18.30	17.90	17.80	100.40	92.80	95.60
	5.60	6.60	4.60	10.80	10.90	11.20	18.30	17.50	19.30	96.40	92.80	103.60
	4.40	7.40	5.50	11.20	11.10	11.90	20.40	19.40	19.80	88.00	84.00	95.60
	5.20	6.90	5.90	8.60	10.20	10.50	19.40	18.00	19.10	87.20	96.80	97.60
	5.40	6.70	4.50	8.90	11.10	13.90	20.30	17.90	18.20	98.40	96.40	101.60
	6.60	6.20	4.00	10.40	12.20	14.50	18.60	17.30	19.60	83.20	94.80	100.80
	3.50	3.90	3.80	8.40	7.80	12.20	18.70	16.60	19.80	84.00	77.20	92.00
Mean	5.87	6.43	5.70	9.39	10.81	11.30	20.12	18.97	19.81	96.35	93.42	93.30
SD	0.90	0.89	1.18	1.39	1.45	1.30	2.13	1.76	1.25	8.13	8.36	6.41
%CV	15.30	13.80	20.70	14.80	13.40	11.50	10.60	9.30	6.31	8.44	8.95	6.87
Grand mean	6.00			10.50			19.63			94.36		
Grand SD	1.03			1.59			1.79			7.69		
Min.	3.50			6.80			16.20			76.40		
Max.	9.00			14.50			24.80			109.20		
n	63			63			63			63		
Grand %CV	17.16			15.14			9.12			8.15		

[a] All results in ppb.

antibody-based methods for qualitatively detecting aflatoxin B_1 in various commodities. These qualitative competitive ELISA methods achieved First Action approval almost 20 years ago and have undergone several changes since their original approval. Several chromatographic methods are also AOAC *Official Methods*[SM]; however, because of their disadvantages, such as high instrumentation costs, need for extensive cleanup, and lengthy analysis time, these methods are not favorable for the rapid testing needs of food and feed industry. Rapid and easy-to-use methods are needed to meet these industry demands. One such method is the Veratox aflatoxin test kit, which was developed by Neogen and has been cited in several peer-reviewed publications (5–10).

3.1 General Information

Aflatoxins are toxic and carcinogenic substances produced by certain strains of the molds *Aspergillus flavus* and *A. parasiticus*. There are four principle types of aflatoxin: B_1, B_2, G_1, and G_2. Aflatoxin B_1 is the most frequently encountered and the most toxic of the group. The commodities most affected by aflatoxin are corn, peanuts, cottonseed, milo, and the majority of tree nuts (11). The effects in animals of ingesting excessive amounts of the toxin

Table 2B. Accuracy and precision of Veratox aflatoxin ELISA[a]

	30 ppb	40 ppb	50 ppb	60 ppb
1	27.2	39.3	50.2	59.8
2	28.9	41.4	48.4	54.8
3	31.0	38.1	46.4	62.6
4	32.1	40.1	47.2	58.8
5	31.3	39.3	52.2	54.6
6	30.5	39.5	46.4	59.4
7	30.0	37.2	56.0	59.2
8	29.4	32.1	39.8	72.4
9	31.5	38.3	46.2	64.6
10	27.9	38.6	50.8	68.6
11	29.9	38.8	53.0	66.6
12	31.6	36.5	51.4	71.4
13	32.1	40.4	50.8	61.8
14	29.9	41.1	53.0	62.6
15	32.3	37.4	53.6	59.2
16	35.4	45.4	48.8	66.0
17	32.0	45.0	56.6	65.8
18	34.5	48.6	51.8	64.0
19	28.2	41.8	57.0	67.0
20	27.2	42.1	45.6	66.4
Mean	30.65	40.05	50.26	63.28
SD	2.20	3.55	4.26	4.91
% CV	7.18	8.86	8.48	7.77
Overall %CV	8.07			

[a] All results in ppb.

range from chronic health and performance problems to death. Aflatoxin has been shown to cause liver damage or cancer, decreased milk and egg production, immune suppression, and interference with reproductive efficiency. The FDA has set maximum allowable levels of aflatoxin in food and feed. Therefore, accurate determination of the presence of the toxin is of major importance to those monitoring the quality of food and feed in which aflatoxin may occur. Testing these commodities for the toxin requires careful sampling, chemical extraction, and quantitative analysis.

3.2 Principle

Veratox for aflatoxin is a direct competitive ELISA in a microwell format that allows the user to obtain exact concentrations in parts per billion (ppb). Free aflatoxin in the samples and controls is allowed to compete with enzyme-labeled aflatoxin (conjugate) for the antibody-binding sites. After a wash step, a substrate is added, which reacts with the bound conjugate to produce a blue color. More blue color means less aflatoxin. The test is read in a microwell reader to yield optical densities. The optical densities of the controls form the standard curve, and the sample optical densities are plotted against the curve to calculate the exact concentration of aflatoxin.

4 Materials and Method

4.1 Test Kit Information

(**4.1.1**) *Kit name*.—Veratox Quantitative ELISA for Aflatoxin.

(**4.1.2**) *Cat. No*.—8030.

(**4.1.3**) *Ordering information.—Inside the United States*.—Neogen Corp., 620 Lesher Pl, Lansing, MI 48912, Tel: 517-372-9200, Fax: 517-372-0108, www.neogen.com. *Outside the United States*.—Contact above for local distributor information.

(**4.1.4**) *Test kit reagents.*

(**4.1.5**) *Antibody-coated microwells (48).*

(**4.1.6**) *Red-marked mixing wells (48).*

(**4.1.7**) *Four yellow-labeled bottles of 1.2 mL each*.—0.0, 5.0, 15.0, and 50 ppb aflatoxin standards in 70% methanol.

(**4.1.8**) *One blue-labeled bottle of 8 mL aflatoxin–horseradish peroxidase conjugate solution.*

(**4.1.9**) *One green-labeled bottle of 12.5 mL K-Blue substrate solution (tetramethylbenzidine).*

(**4.1.10**) *One red-labeled bottle of 12.5 mL Red Stop solution (0.0006% NaF + cresol red).*

4.2 Additional Supplies and Reagents (Required but Not Included in the Test Kit)

(**4.2.1**) *Extraction materials (items **4.2.1.1** through **4.2.1.5** available in kit form from Neogen, item No. 8052).*

(**4.2.1.1**) *70% ACS grade methanol (Neogen item No. 8055).*

(**4.2.1.2**) *250 mL graduated cylinder (Neogen item No. 9368).*

(**4.2.1.3**) *Container with 250 mL capacity*.

(**4.2.1.4**) *Neogen filter syringes, Whatman No. 1 filter paper, or equivalent*.

(**4.2.1.5**) *Sample collection tubes 12 75 mm*.

(**4.2.2**) *Paper towels or equivalent absorbent material.*

(**4.2.3**) *Plastic bucket for use as waste receptacle*.

(**4.2.4**) *Microwell holder (Neogen item No. 9402).*

(**4.2.5**) *Timer (Neogen item No. 9426*).

(**4.2.6**) *Waterproof marker*.

(**4.2.7**) *Wash bottle (Neogen item No. 9400).*

(**4.2.8**) *Two reagent boats for 12-channel pipettor (Neogen item No. 9435).*

(**4.2.9**) *Distilled or deionized water*.

4.3 Apparatus

(**4.3.1**) *Scale capable of weighing 5–50 g (Neogen item No. 9427*).

(**4.3.2**) *Microwell reader with a 650 nm filter or equivalent with Neogen Veratox software*.

(**4.3.3**) *12-channel pipettor (Neogen item No. 9273).*

Table 2C. Shake versus blend extraction[a]

	NDA		5.7 ppb		9.2 ppb		18.0 ppb		111.88 ppb	
	Shake	Blend	Shake	Blend	Shake	Blend	Shake	Blend	Shake	Blend
	0.40	0.40	5.70	5.20	11.60	8.40	22.30	20.40	105.20	90.50
	0.20	0.30	6.20	5.30	9.70	10.10	20.60	21.20	107.20	85.50
	0.80	0.00	6.30	5.30	10.20	10.90	19.00	18.00	101.20	96.00
	0.90	0.50	7.20	6.20	9.50	11.50	22.50	18.90	109.20	87.40
	1.60	0.00	5.90	5.20	9.10	10.70	23.60	21.70	106.40	83.80
	0.30	0.40	5.80	4.10	10.30	9.90	24.80	17.80	104.00	90.90
	0.40	0.20	6.50	6.40	8.10	11.00	23.40	19.40	98.00	92.50
	0.30	0.60	7.00	5.90	10.30	7.40	18.10	24.10	94.80	90.00
	0.50	0.00	6.60	5.00	8.40	10.80	20.10	19.60	91.20	123.50
	1.00	0.00	5.50	5.30	11.60	8.80	19.40	20.20	98.40	102.60
Mean	0.64	0.24	6.27	5.39	9.88	9.95	21.38	20.13	101.56	94.27
SD	0.44	0.23	0.56	0.65	1.18	1.33	2.25	1.88	5.86	11.58
%CV	NA	NA	8.96	12.13	11.94	13.38	10.50	9.33	5.77	12.29

[a] All results in ppb.

(**4.3.4**) *100 L pipettor (Neogen item No. 9272).*

(**4.3.5**) *Tips for 100 L and 12-channel pipettors (Neogen item No. 9410).*

4.4 Certified Reference Materials (Trilogy Analytical Laboratories)

All samples were naturally contaminated North American corn. Samples were prepared by Trilogy Analytical Laboratories (Washington, MO) according to specifications set forth in the USDA-GIPSA documentation (12). Four corn samples naturally contaminated with aflatoxins at 5, 10, 20, and 100 ppb (±15%) were ground (2 kg each) so that 95% of the material passed through a 20-mesh sieve. The corn samples at each concentration level were then divided into seven sets of 50 g subportions and run by the HPLC reference method three times each (Table 1). A 2 kg portion of aflatoxin-free corn was also prepared under the same conditions and verified as <1 ppb by the HPLC reference method. Spike material used for robustness portion of the study was total aflatoxin (B_1:B_2:G_1:G_2) prepared to a ratio of (10:1:1:1), respectively, in 100% methanol and also prepared by Trilogy Analytical Laboratories.

4.5 Sample Preparation

(**4.5.1**) All samples were blind-coded.

(**4.5.2**) Samples were stored at 2–8 C until analyzed.

(**4.5.3**) *Sample extraction.*

(**4.5.3.1**) Add 50 g ground corn sample to a clean disposable extraction bottle containing 250 mL 70% methanol.

(**4.5.3.2**) Tightly cap and vigorously shake the bottle for 3 min.

(**4.5.3.3**) Allow the solids to settle to the bottom of the bottle for about 1 min.

(**4.5.3.4**) Filter the contents through folded filter paper or Neogen filter syringe into a clean container. The sample is now ready for testing.

Table 3. Direct comparison of HPLC to Veratox aflatoxin ELISA[a, b]

HPLC	ELISA
0.0	0.0
5.6	5.4
8.1	9.2
10.0	10.2
13.0	15.7
14.1	12.7
17.1	15.8
19.4	19.8
20.4	24.8
20.7	23.5
22.6	22.9
24.9	32.0
33.8	32.1
38.5	28.9
70.1	80.7

[a] *n* = 3 for each sample (averages shown). LOD for HPLC = 0.1 ppb. All results in ppb.

[b] LOD for **994.08** = 0.1 ppb aflatoxin B1. LOD for Veratox method = 1.4 ppb total aflatoxin.

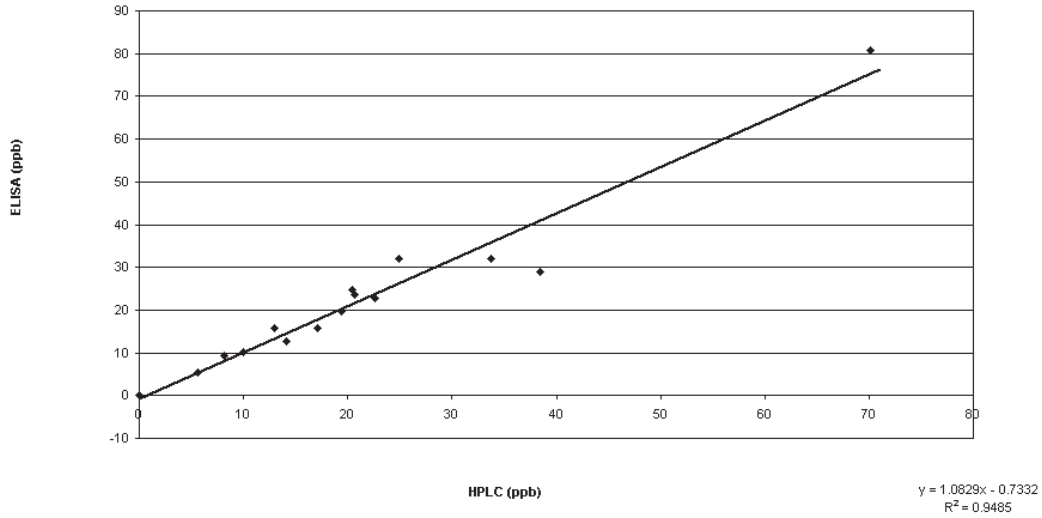

Figure 1. HPLC versus ELISA.

4.6 Procedural Notes

K-Blue substrate is ready for use.—The substrate should be clear to light blue. Discard if it has turned dark blue. Pour only the needed volume of substrate into a reagent boat. Do not return unused substrate to the bottle. Cover the reagent boat to keep the substrate protected from light until it is needed.

Antibody wells.—Keep wells sealed in the foil pouch until needed. Remove wells from the foil pouch only after samples are extracted, and the test is set to begin.

4.7 Precautions

(**4.7.1**) Methanol solution is highly flammable. Keep container tightly closed, and keep away from heat, sparks, open flame, and those smoking. It is toxic if swallowed, or if vapor is inhaled. Avoid contact with skin.

(**4.7.2**) Store test kit between 2–8 C when not in use. Do not freeze.

(**4.7.3**) Kits should be brought to room temperature (18–30 C) before use.

(**4.7.4**) Avoid prolonged storage of kits at ambient temperatures for more than 8 h per day without returning to refrigerator in between.

(**4.7.5**) Do not use kit components beyond expiration date.

(**4.7.6**) Do not mix reagents from one kit serial with reagents from a different kit serial.

(**4.7.7**) Do not run more than 24 wells at a time.

(**4.7.8**) Follow proper pipetting techniques, including priming of tips.

(**4.7.9**) Use of incubation times other than those specified may give inaccurate results.

(**4.7.10**) Treat all used liquids, including sample extract, and labware as if contaminated with aflatoxin. Gloves and other protective apparel should be worn at all times.

4.8 Analysis

(**4.8.1**) Remove one red-marked mixing well for each sample to be tested plus four red-marked wells for controls, and place in the well holder.

(**4.8.2**) Remove an equal number of antibody-coated wells. Return antibody wells that will not be used immediately to the foil pack with desiccant. Reseal the foil pack to protect the antibody. Mark one end of strip with a "1," and place strip in the well holder with the marked end on the left. Do not mark the inside or bottom of the wells.

(**4.8.3**) Mix each reagent by swirling the reagent bottle before use.

(**4.8.4**) Place 100 L of conjugate from the blue-labeled bottle in each red-marked mixing well.

(**4.8.5**) Using a new pipet tip for each, transfer 100 L of controls and samples to the red-marked mixing wells as described below:

0	5	15	50	S1	S2	S3	S4	S5	S6	S7	S8	Strip 1	
S9	S10	S11	S12	S13	S14	S15	S16	S17	S18	S19	S20	Strip 2	

(**4.8.6**) Using a 12-channel pipettor, mix the liquid in the wells by pipetting it up and down three times. Transfer 100 L to the antibody-coated wells. Discard the red-marked mixing wells.

(**4.8.7**) Set timer for 2 min, mixing the wells for the first 10–20 s of the room temperature incubations by sliding the microwell holder back and forth on a flat surface without splashing reagents from wells.

(**4.8.8**) Shake out the contents of the antibody wells. Fill the wells with distilled or deionized water and dump them out. Repeat this step five times; then turn the wells upside-down and tap out on a paper towel until the remaining water has been removed.

(**4.8.9**) Pour the needed volume of substrate from the green-labeled bottle into the green-labeled reagent boat.

Table 4. Cross-reactivity testing of Veratox antibody

Cross-reactive compound	Cross-reactivity, %
Aflatoxin B_1	100
Aflatoxin B_2	31
Aflatoxin G_1	28
Aflatoxin G_2	3.8
Total fumonisins	<0.01
T2-toxin	<0.01
Ochratoxin	<0.01
Zearalenone	<0.01
Deoxynivalenol	<0.01

(**4.8.10**) With new tips on the 12-channel pipettor, prime and pipet 100 L of substrate into the wells.

(**4.8.11**) Set timer for 3 min, mixing the wells for the first 10–20 s by sliding back and forth on a flat surface. Discard remaining substrate and rinse the reagent boat with water.

(**4.8.12**) Pour Red Stop solution from the red-labeled bottle into the red-labeled reagent boat.

(**4.8.13**) Eject the excess substrate from the 12-channel pipettor, prime the tips, and pipet 100 L of Red Stop to each well. Mix by sliding back and forth on a flat surface for 10–20 s. Discard the tips.

(**4.8.14**) Wipe the bottom of the microwells with a dry cloth or towel and read in a microwell reader using a 650 nm filter. Air bubbles should be avoided through careful pipetting, as they could affect analytical results. Results should be read within 20 min after the addition of Red Stop.

(**4.8.15**) Read and calculate results using Neogen's Stat Fax microwell reader, or equivalent. If using an equivalent reader, calculate results using Neogen's Veratox software. Data are considered invalid if correlation coefficient of standard curve is <0.98.

4.9 Interpretation of Results

Results between 5.0 and 50 ppb are quantitative. Results >50 ppb should be diluted for proper quantitation.

5 Safety Precautions

Treat sample extract and labware as if they contain aflatoxin to avoid contamination of test samples and the laboratory environment. Soak all used laboratory ware, pipet tips, and kit components in a 10% solution of household bleach before discarding.

6 Internal Validation Studies

All analyses will be generated from one kit lot unless specified below.

6.1.1 Accuracy

Twenty-one blind-coded samples were prepared for each of four naturally contaminated aflatoxin levels (5.7, 9.2, 18.0, and 111.8 ppb). Each analyst extracted seven samples of each level, and each sample extract was independently analyzed by all three individual technicians in the same laboratory using the same reader.

(**6.1.1.1**) *Testing*.—The above preparations were tested following procedures set forth above.

(**6.1.1.2**) *Results*.—The evaluation of sample extracts for accuracy indicates 105.3% recovery at 5.7 ppb, 114.1% recovery at 9.2 ppb, 109.1% recovery at 18.0 ppb, and 84.4% recovery at 111.8 ppb in corn (Table 2A). Recovery was calculated by comparison to the mean of the reference method result. An overall 103.2% recovery was observed across all levels.

(**6.1.2**) Twenty blind-coded samples were prepared for each of four fortified aflatoxin levels (30.0, 40.0, 50.0, and 60.0 ppb) in previously tested no detectable amount (NDA) corn. Each sample extract was analyzed by a single technician. NDA corn was spiked using reference material described above.

(**6.1.2.1**) *Testing*.—The above preparations were tested following procedure set forth above.

(**6.1.2.2**) *Results*.—The evaluation of sample extracts for accuracy indicates 102.2% recovery at 30.0 ppb, 100.1% recovery at 40 ppb, 100.5% recovery at 50 ppb, and 105.5% recovery at 60 ppb in corn (Table 2B). An overall 102.1% recovery was observed across all levels, indicating acceptable linearity over the central range of the standard curve.

(**6.1.3**) *Blend versus shake extraction.*—Two sets of 10 blind-coded samples were prepared for each of four naturally contaminated aflatoxin levels (5.7, 9.2, 18.0, and

Table 5. Limit of detection using aflatoxin-free corn[a]

Replicate	Veratox	HPLC
1	0.4	NDA[b]
2	0.2	NDA
3	0.8	NDA
4	0.9	NDA
5	1.6	NDA
6	0.3	NDA
7	0.4	NDA
8	0.3	NDA
9	0.5	NDA
10	1.0	NDA
Mean	0.6	<0.01
SD	0.4	NA
Mean + 2SD	1.4	NA

[a] LOD of HPLC = 0.1 ppb; all values in ppb.
[b] NDA = No detectable amount.

Table 6. Veratox aflatoxin robustness analysis: temperature[a]

	Temperature, C		
Level, ppb	18	24	30
	Day 1		
0	0.3	1.0	1.6
0	0.8	1.1	1.9
0	0.6	0.7	1.4
15	17.9	20.2	17.6
15	17.4	18.5	19.0
15	17.4	15.6	16.5
25	24.7	25.6	26.6
25	28.3	26.0	25.7
25	28.4	28.8	26.5
R^2	0.999	0.995	0.995
	Day 2		
0	0.5	0.6	1.0
0	0.8	0.3	1.1
0	0.7	0.2	0.4
15	14.9	13.7	14.5
15	17.2	14.1	12.7
15	17.6	12.3	13.5
25	30.4	27.2	28.3
25	29.3	26.7	28.6
25	33.9	33.5	36.3
R^2	0.997	0.995	0.997

[a] All results in ppb.

Table 7. Veratox aflatoxin robustness analysis: reagent volume[a]

	Volume, L		
Level, ppb	50	100	150
	Day 1		
0	0.1	0.3	1.6
0	0.4	0.5	1.9
0	0.0	0.3	1.8
15	23.7	15.6	17.7
15	23.0	17.7	16.4
15	19.3	16.3	15.8
25	31.0	26.6	24.4
25	30.5	24.4	24.1
25	28.8	27.2	27.8
R^2	0.991	0.994	0.999
	Day 2		
0	0.1	0.7	0.3
0	0.3	0.4	0.3
0	0.4	0.8	0.1
15	14.5	14.5	13.0
15	15.0	15.5	15.3
15	15.3	16.1	14.3
25	32.5	27.8	24.8
25	31.4	28.0	25.2
25	29.2	30.6	28.4
R^2	0.999	0.994	0.999

[a] All results in ppb.

111.8 ppb) and NDA. Each sample extract was analyzed by one technician.

(**6.1.3.1**) *Testing*.—Set 1 of the above preparations was tested following procedures set forth above, and Set 2 followed all procedures stated above, with the exception that a 1 min high-speed blend extraction was substituted for the purposes of comparing efficiencies.

(**6.1.3.2**) *Results*.—The evaluation of sample extracts for purposes of comparison of a shake versus blender extraction indicates no appreciable difference in accuracy or reproducibility based on extraction scheme used (Table 2C).

6.2 Precision

Twenty-one blind-coded samples were prepared for each of four naturally contaminated aflatoxin levels (5.7, 9.2, 18.0, and 111.8 ppb). Each analyst extracted seven samples of each level, and each sample extract was independently analyzed by all three individual technicians in the same laboratory using the same reader.

(**6.2.1**) *Testing*.—The above preparations were tested following procedures set forth above.

(**6.2.2**) *Results*.—The evaluation of sample extracts for precision indicates a 17.16% CV at 5.7 ppb, a 15.14% CV at 9.2 ppb, a 9.12% CV at 18.0 ppb, and an 8.15% CV at 111.8 ppb in corn as illustrated in Table 2A. An overall 12.39% CV was observed across all levels (Table 2A).

6.3 Comparison to Existing Methods

Blind-coded duplicate samples were analyzed by the ELISA and HPLC methods using the AOAC reference method and reported in ppb. All samples were analyzed in triplicate and means were compared.

(**6.3.1**) *Testing*.—The above preparations were tested following procedures set forth above for the Neogen method. The AOAC reference method was performed according to *Official Method* **994.08**.

(**6.3.2**) *Results*.—Data indicate 0.9485 correlation between the ELISA method and the reference method (Table 3). Scatter plot shows a linear relationship with a slope of 1.0829 (Figure 1).

Table 8. Veratox aflatoxin robustness analysis: number of washes[a]

	Volume		
Level, ppb	2	5	10
	Day 1		
0	0.9	0.6	1.5
0	1.1	1.4	2.1
0	0.9	0.8	1.8
15	16.3	18.4	23.2
15	17.6	18.1	23.1
15	16.1	17.5	23.0
25	24.8	26.5	33.1
25	24.0	25.6	30.8
25	27.2	27.4	29.6
R^2	0.998	0.996	0.993
	Day 2		
0	0.1	0.1	0.8
0	0.1	0.2	0.7
0	0.1	0.0	0.1
15	9.2	13.2	14.5
15	10.4	14.4	15.6
15	10.1	12.8	14.1
25	25.5	27.9	28.2
25	26.6	28.4	31.2
25	29.8	28.5	31.4
R^2	0.983	0.999	1.000

[a] All results in ppb.

Table 9. Veratox aflatoxin robustness analysis: shaking time[a]

	Shake time, s		
Level, ppb	5	20	40
	Day 1		
0	2.0	0.3	0.2
0	2.6	0.0	0.7
0	2.5	0.0	0.5
15	19.5	15.9	17.4
15	18.3	14.7	16.6
15	17.6	14.9	16.6
25	26.5	23.8	27.1
25	25.6	23.8	24.1
25	29.7	25.4	29.3
R^2	0.998	0.995	0.998
	Day 2		
0	0.4	0.5	0.3
0	0.1	0.5	0.8
0	0.4	0.3	0.4
15	11.1	16.3	14.7
15	12.4	15.2	15.6
15	10.9	14.7	16.2
25	26.3	26.5	27.5
25	27.0	27.5	26.8
25	31.6	30.3	32.1
R^2	0.995	0.998	0.994

[a] All results in ppb.

6.4 Cross-Reactivity

Data were generated quantifying cross-reactivity for the Veratox aflatoxin kit with various aflatoxins and other mycotoxins. Potential cross-reactive compounds tested included aflatoxin B_1, B_2, G_1, and G_2, as well as ochratoxin, fumonisin (total), deoxynivalenol, T-2 toxin, and zearalenone. Various compounds were prepared in 70% methanol and analyzed with the test kit.

(**6.4.1**) *Preparation of cross-reactors*.—Each potential cross-reactive compound was purchased as a certified reference solution from Trilogy Analytical Laboratories.

(**6.4.2**) *Testing*.—Each compound was diluted in 70% methanol and evaluated in duplicate in the same manner as a standard sample.

(**6.4.3**) *Results*.—Cross-reactivity was not observed on any of the above listed nonaflatoxin compounds. Aflatoxins (B_1, B_2, G_1, and G_2) showed a profile of 100, 31, 28, and 3.8%, respectively. Percent recoveries were calculated against aflatoxin B_1. Compounds listed as <0.01% cross-reactivity elicited no response at a spike level of 10 000 ng/mL (Table 4).

6.5 Detection Limit

Data are submitted showing the detection limit on one sample of aflatoxin-free corn tested 10 times. The detection limit is expressed as the mean value of the negative sample determination plus two SD values. Negative samples were confirmed as such by Trilogy Analytical Laboratories using AOAC *Official Method*℠ **994.08**.

(**6.5.1**) *Testing*.—The above preparations were tested following procedures set forth above.

(**6.5.2**) *Results*.—Detection limit was determined to be 1.4 ppb for corn by ELISA (Table 5).

6.6 Ruggedness Testing

Data are included demonstrating ruggedness based on triplicate assays of three spiked samples of NDA corn (0, 15, and 25 ppb) tested over three conditions each, over five different parameters each, over 2 days. The three conditions represent a low, median, and high value of each parameter.

Table 10. Veratox aflatoxin robustness analysis: incubation time[a]

	Incubation time, min		
Level, ppb	1.5	2.0	2.5
	Day 1		
0	1.0	0.3	0.4
0	0.9	0.3	0.9
0	0.5	02	0.7
15	19.9	13.5	14.9
15	16.1	16.8	18.2
15	16.3	14.2	17.4
25	25.5	23.9	24.5
25	25.1	21.2	23.7
25	26.0	30.4	27.8
R^2	0.989	0.992	0.998
	Day 2		
0	0.2	0.0	0.3
0	0.7	0.0	0.8
0	0.3	0.0	0.0
15	12.5	12.0	14.9
15	13.3	11.3	14.5
15	16.2	13.2	13.5
25	26.9	28.8	28.4
25	29.3	26.1	28.1
25	29.3	32.6	27.9
R^2	0.994	0.999	1.000

[a] All results in ppb.

Table 11. Veratox aflatoxin robustness analysis: sample extraction shaking intensity[a]

	Extraction intensity, rpm		
Level, ppb	150	110	75
	Day 1		
0	1.0	0.1	0.2
0	1.1	0.6	0.4
0	0.7	0.7	0.6
15	20.2	14.6	17.8
15	18.5	16.4	18.6
15	15.6	17.2	18.2
25	25.6	26.1	25.2
25	26.0	26.5	28.4
25	28.8	26.5	26.8
R^2	0.995	1.00	0.997
	Day 2		
0	0.6	0.5	0.9
0	0.3	0.8	0.7
0	0.2	0.9	1.2
15	13.7	14.8	16.8
15	14.1	13	18.2
15	12.3	17.2	18.1
25	27.2	25.2	27.0
25	26.7	25.0	29.2
25	33.5	24.3	28.2
R^2	0.995	0.999	0.999

[a] All results in ppb.

Parameters included temperature, shaking time, reagent volume, number of washes, incubation time, and extraction shaking intensity. NDA corn was spiked using reference material described above. All samples were blind-coded.

(**6.6.1**) *Temperature*.—All parameters of the kit were observed as indicated above, with the exception of assay temperature, which was intentionally fluctuated to include 18, 24, and 30 C. These temperature fluctuations appear to have little effect on results when compared to the optimal temperature of 24 C. To obtain consistently acceptable correlation coefficients, it is recommended to perform the assay at temperatures above 20 C (Table 6).

(**6.6.2**) *Reagent volume*.—All parameters of the kit were observed as indicated above, with the exception of reagent volume for both competition and substrate steps, which were intentionally fluctuated to include 50, 100, and 150 L. Day 1 data indicate that lower assay volume may have the potential to generate inflated results. Based on data, an optimal volume of 100 L is recommended (Table 7).

(**6.6.3**) *Number of washes*.—All parameters of the kit were observed as indicated above, with the exception of number of washes, which was intentionally fluctuated to include two, five, and 10 washes. These fluctuations appear to have little effect on results. Although little effect was observed, an optimum number of five washes is still recommended (Table 8).

(**6.6.4**) *Shaking time*.—All parameters of the kit were observed as indicated above, with the exception of shaking time for both competition and substrate steps, which were intentionally fluctuated to include 5, 20, and 40 s. These fluctuations appear to have little effect on results. To obtain consistently acceptable correlation coefficients, it is recommended to perform the assay using the optimal shaking time of 20 s (Table 9).

(**6.6.5**) *Incubation time*.—All parameters of the kit were observed as indicated above, with the exception of incubation time for both competition and substrate steps, which were intentionally fluctuated to include 1.5, 2, and 2.5 min. These fluctuations appear to have little effect on results. Although little effect was observed, an optimum timing of 2–3 min per step is still recommended (Table 10).

(**6.6.6**) *Extraction shaking intensity*.—All parameters of the kit were observed as indicated above. Samples normally extracted using a mechanical shaker at 150 rpm for 3 min were

Table 12. Lot-to-lot variability[a]

	Lot-to-lot No.		
	15213 (7/22/08)	15220 (11/13/08)	15225 (1/28/09)
	Days to expiration		
Level, ppb	32	111	218
0	0.2	0.4	0.0
0	0.3	0.8	0.0
0	0.3	1.2	0.2
15	12.0	9.7	11.9
15	12.7	12.1	12.5
15	11.5	12.0	12.5
25	28.4	24.9	28.7
25	21.4	23.4	28.6
25	33.1	24.7	28.1
R^2	0.987	0.999	0.991

[a] All results in ppb.

also intentionally extracted using ¾ and ½ speed in order to observe the effects. These fluctuations appear to have little effect on results (Table 11).

6.7 Lot-to-Lot Variability and Stability

Lot-to-lot variability is assessed by testing samples of spiked NDA corn at three levels (0, 15, and 25 ppb). Results were taken from a standard curve. Assays were performed in triplicate for each of three different kit lots. The three kits lots represent reagents at the beginning, middle, and end of usable shelf life.

(**6.7.1**) *Testing*.—The above preparations were tested following procedures set forth above.

(**6.7.2**) *Results*.—Results generated from each lot of kits were found to be comparable (Table 12), although acceptable correlation coefficients became more difficult to obtain toward the end of shelf life.

6.8 Additional Commodities Testing

Data are included demonstrating applicability of the test method on commodities other than corn. NDA commodities were spiked using reference material described above. Five blind-coded samples were prepared for each of two fortified aflatoxin levels (5.0 and 20.0 ppb) in previously tested NDA commodities. Each sample extract was analyzed by a single technician. Commodities selected were popcorn, wheat, soybeans, cottonseed, sorghum, millet, and dried distillers grains with solubles. Ten blind-coded samples were also prepared for each commodity unfortified for demonstration of acceptable LOD. Each sample extract was analyzed by a single technician.

(**6.8.1**) *Testing*.—The above preparations were tested following the procedure set forth above.

Table 13. Results of independent laboratory trials with the Veratox aflatoxin ELISA[a, b]

	5.0 ppb	20.0 ppb
	Analyst A	
1	3.5	20.9
2	4.3	20.7
3	4.7	20.8
4	5.7	26.2
5	9.0	25.8
6	7.1	25.0
7	6.6	26.7
	Analyst B	
1	6.0	18.1
2	5.0	19.9
3	6.5	18.3
4	5.6	24.0
5	6.2	25.1
6	7.8	24.2
7	6.8	33.0
	Analyst C	
1	6.5	22.8
2	6.6	22.2
3	6.2	21.4
4	6.5	18.7
5	7.1	24.8
6	8.1	23.8
7	7.2	26.9
Mean	6.3	23.3
SD	1.3	3.6
CV, %	20.1	15.2

[a] All results in ppb.

[b] HPLC Method **994.08** also performed by independent laboratory: result for 5 ppb sample = 5.83 ppb; result for 20 ppb sample = 20.69 ppb.

(**6.8.2**) *Results*.—Although this method is not seeking approved status for commodities other than corn at this time, data support a future method modification provided a statistically significant study is performed (Appendix A).

6.9 Independent Laboratory Validation

In accordance with Federal Grain Inspection Service (FGIS) guidelines, the Veratox aflatoxin test kit was submitted to the USDA-GIPSA Technical Services Division, Kansas City, MO, for independent analysis. Seven blind-coded naturally contaminated samples at 5 ng/g and an additional seven at 20 ng/g were analyzed by three technicians. Results and statistical analysis are shown in Table 13. To be granted GIPSA-approved status, 20–21 results must be within acceptable range, defined as 5.0 ± 3.0 and 20.0 ± 10.0 ppb.

(**6.9.1**) *Testing*.—The above preparations were tested following the procedure set forth above.

(**6.9.2**) *Results*.—Results generated were found to meet GIPSA performance criteria and correlated well with reference methods (Table 13).

7 Conclusions

Given the observed accuracy over several levels of naturally contaminated reference corn compared to HPLC and the degree of reproducibility observed in both the internal and collaborating laboratory data set, it is recommended that the Veratox for aflatoxin method be granted *Performance Tested Method* status for corn.

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Appendix A. Additional commodities

Replicate	Popcorn	Wheat	Soybeans	Cottonseed	Sorghum	Dried distillers grains	Millet
			Unfortified				
1	0.90	0.00	0.00	1.90	0.00	2.00	0.20
2	1.20	0.00	0.00	1.50	0.00	1.90	1.00
3	1.60	0.00	0.10	1.90	0.00	1.50	1.30
4	2.10	0.00	0.00	2.10	0.10	1.30	1.50
5	0.40	0.00	0.00	2.00	0.50	0.90	0.90
6	0.20	0.00	0.00	1.20	0.80	1.30	1.00
7	0.40	0.00	0.00	2.00	0.00	1.90	0.50
8	0.00	0.00	0.00	1.10	0.20	0.70	0.00
9	0.50	0.00	0.00	2.00	0.50	1.80	0.00
10	1.40	0.00	0.40	3.30	0.20	2.10	0.20
Mean	0.87	0.00	0.05	1.90	0.23	1.54	0.66
SD	0.68	0.00	0.13	0.61	0.28	0.48	0.55
Mean + 2SD	2.24	0.00	0.30	3.11	0.79	2.50	1.76
			Fortified with 5 ppb aflatoxin				
1	6.00	3.80	5.00	5.60	5.00	6.30	2.60
2	6.00	3.70	4.90	5.70	4.80	6.10	2.40
3	6.00	2.80	4.10	5.20	4.20	6.50	2.80
4	6.30	3.60	4.40	5.30	3.80	6.70	3.60
5	6.50	3.90	4.20	5.60	4.30	6.30	4.40
Mean	6.16	3.56	4.52	5.48	4.42	6.38	3.16
SD	0.23	0.44	0.41	0.22	0.48	0.23	0.83
			Fortified with 20 ppb aflatoxin				
1	26.40	16.80	19.70	14.40	22.10	18.80	19.10
2	25.60	17.80	17.70	11.90	20.60	17.60	17.00
3	25.70	16.90	19.00	11.80	21.40	17.40	17.90
4	29.70	17.90	19.50	11.80	20.70	17.60	16.30
5	29.00	16.70	19.70	11.20	20.50	16.00	13.90
Mean	27.28	17.22	19.12	12.22	21.06	17.48	16.84
SD	1.93	0.58	0.84	1.25	0.68	1.00	1.95