Intended Use

The AgraQuant® Total Aflatoxin Assay is a direct competitive enzyme-linked immunosorbent assay (ELISA) that determines a quantitative level for the presence of total aflatoxin (B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub> and G<sub>2</sub>) and is intended for use in grains, cereals, nuts, animal feeds and other commodities.

The AgraQuant® Total Aflatoxin Assay 1/20 has been validated for almond, beer, canola oil, chilli, corn, malted barley and pistachio.

Aflatoxins

Aflatoxins are toxic and carcinogenic. They are metabolites of the fungi Aspergillus flavus and Aspergillus parasiticus. There are four principle types of aflatoxin: B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub> and G<sub>2</sub>, which are named for their respective innate fluorescent properties. Aflatoxin B<sub>1</sub> is the most frequently encountered of the group and the most toxic. Aflatoxins can be found mainly in cereals, corn, peanuts, cottonseed and nuts.

Aflatoxins can cause liver disease in animals and may cause decreased production (milk, eggs, animal weight, etc). Aflatoxin B<sub>1</sub> is a potent human carcinogen, and may contribute to human liver cancer.

The US Food and Drug Administration action levels of aflatoxin are as follows: (1) 300ppb for feeder cattle; (2) 200ppb for finishing swine; (3) 100ppb for breeding beef cattle, swine and mature poultry; and (4) 20ppb for humans, and for immature animals and dairy animals.

Assay Principles

The AgraQuant® Total Aflatoxin Assay is a direct competitive enzyme-linked immunosorbent assay (ELISA). Aflatoxins are extracted from a ground sample with 70% methanol. The extracted sample and enzyme-conjugated aflatoxin are mixed and added to the antibody-coated microwell. Aflatoxins in samples and control standards are allowed to compete with enzyme-conjugated aflatoxin for the antibody binding sites. After a washing step, an enzyme substrate is added and blue color develops. The intensity of the color is inversely proportional to the concentration of aflatoxin in the sample or standard. A stop solution is then added which changes the color from blue to yellow. The microwells are measured optically using a microwell reader with an absorbance filter of 450nm (OD<sub>450</sub>) and a differential filter of 630nm. The optical densities of the samples are compared to the OD’s of the standards and an interpretative result is determined.

Precautions

1. Store reagents at 2-8°C (35-46°F) when not in use, and do not use beyond the expiration date.
2. Adhere to incubation times stated in the procedure. Use of incubation times other than those specified may give inaccurate results.
3. Methanol is flammable. Caution must be taken in its use and storage.
4. The Stop Solution contains acid. Avoid contact with skin or eyes. If exposed, flush with water.
5. Consider all materials, containers and devices that are exposed to the sample or standards to be contaminated with toxin. Wear protective gloves and safety glasses when using the kit.
6. Dispose of all materials, containers and devices appropriately after use.
**Procedure**

**Sample Preparation / Extraction**

1. Obtain a representative sample and grind it using a Romer Series II® Mill so that 75% will pass through a 20-mesh screen, then thoroughly mix the subsample portion.
2. Weigh out 20 g of ground sample into a clean jar that can be tightly sealed.
3. Add 100 mL of 70/30 (v/v) methanol/water extraction solution and seal jar. **Note:** Samples should be extracted in a ratio of 1:5 (w:v) of sample to extraction solution respectively.
4. Vigorous shake or blend for 3 minutes.
5. Allow sample to settle, then filter the top layer of extract through a Whatman #1 filter and collect the filtrate.

**For beer:** measure 3mL of beer sample into a test tube and add 7mL of 100% methanol into the same tube, vortex or mix for 30 seconds. Sample is now ready for testing. The final result of aflatoxin in beer is calculated by multiplying a factor of \((2/3)\) to the ELISA result.

**For canola oil:** measure 2mL of canola oil sample into a test tube and add 10mL of 70% methanol (the extraction ratio is 1:5 of sample to extraction solution); mix or vortex for 3 minutes; allow sample to separate into two layers; take the methanol layer for ELISA testing. (For some oil samples which may not separate into two layers upon standing, please contact technical services).

**For chilli:** weigh out 20g of ground chilli sample; add 100mL of 84/16 (v/v) acetonitrile/water extraction solution; vigorous shake or blend for 3 minutes; allow sample to settle and then filter the top layer of extract through a Whatman#1 filter and collect the filtrate; clean the filtrate with a MycoSep 112 column; take 1mL of cleaned extract and evaporate to dryness; reconstitute it with 1mL of 70% methanol; the sample is now ready for ELISA testing.

**Note:** Commodity extracts should have a pH of 6-8. Excessive alkaline or acidic conditions may affect the test results and should be adjusted before testing.

**Assay**

**Note:** All reagents and kit components must be at room temperature 18-30°C (64-86°F) before use. It is recommended that an 8-channel pipettor be used to perform the assay. No more than 48 samples and standards total (6 test strips) should be run in one experiment when using an 8-channel pipettor. If an 8-channel pipettor is not used (i.e. using only single channel pipettes), it is recommended that no more than a total of 16 samples and standards (2 test strips) be run in any one experiment.

1. Place the appropriate number of blue/green-bordered Dilution Strips in a microwell strip holder. One Dilution Well will be required for each standard, (i.e. 0, 1.0, 2.0, 4.0, 10.0 and 20.0 ppb) or sample.
2. Place an equal number of Antibody Coated Microwell strips in a microwell strip holder. Return unused microwell strips to the foil pouch with the desiccant packet and reseal pouch with tape.
3. Measure the required amount of Conjugate from the green-capped bottle (~240 µL/well or 2 mL/strip) and place in a separate container (e.g. reagent boat when using the 8-channel pipettor). Using an 8-channel pipette, dispense **200 µL of Conjugate** into each blue/green-bordered Dilution Well.
4. Using a single channel pipettor, add **100 µL of each standard or sample** into the appropriate Dilution Well containing 200 µL of Conjugate. Use a fresh pipette tip for each standard or sample. **Note:** Make sure the pipette tip has been completely emptied. Using an 8-channel pipettor with fresh tips for each 8-well strip, mix each well by carefully pipetting it up and down 3 times and immediately transfer **100 µL of the contents from each Dilution Well** into a corresponding Antibody Coated Microwell. Incubate at room temperature for **15 minutes**. **Note:** Do not agitate the plate to mix as it may cause well-to-well contamination.
5. Empty the contents of the microwell strips into a waste container. Wash by filling each microwell with distilled or deionized water, and then dumping the water from the microwell strips. Repeat this step 4 times for a total of 5 washes. **Note:** Take care not to dislodge the strips from the holder during the wash procedure. A piece of tape may be placed on the edge of the holder to help keep strips in place.
6. Lay several layers of absorbent paper towels on a flat surface and tap microwell strips on towels to expel as much residual water as possible after the fifth wash. Dry the bottom of the microwells with a dry cloth or towel.

7. Measure the required amount of Substrate from the blue-capped bottle (~120 µL/well or 1 mL/strip) and dispense into a separate container (e.g. reagent boat for an 8-channel pipettor). Pipette 100 µL of the Substrate into each microwell strip using an 8-channel pipettor. Incubate at room temperature for 5 minutes.

8. Measure the required amount of Stop Solution from the red-capped bottle (~120 µL/well or 1 mL/strip) and dispense into a separate container (e.g. reagent boat for an 8-channel pipettor). Pipette 100 µL of Stop Solution into each microwell strip using an 8-channel pipettor. The color should change from blue to yellow.

9. Read the strips with a microwell reader using a 450 nm filter and a differential filter of 630nm. Record OD readings for each microwell. Note: Air bubbles should be eliminated prior to reading strips as they may affect analytical results.

Additional Notes: Ratio of Conjugate to Standard/Sample should remain at 2:1, but volumes of Conjugate and Standards/Samples can be reduced, e.g. using 100µL and 50µL, respectively. The content to be transferred from dilution well to antibody coated well remains the same as 100 µL. Do not return unused reagents to their original bottles. Carefully keep track of the position of Samples and Standards during the assay. Do not mix the assay microwells by shaking at any time during test.

**Interpretation of the Results**

Using either the unmodified OD values or the OD values expressed as a percentage of the OD of the zero (0) standard, construct a dose-response curve using the five standards. Since the amount of aflatoxin in each standard is known, the unknowns can be measured by interpolation from this standard curve. Results can also be easily calculated using the Romer® Log/Logit spreadsheet that is provided (free of charge) upon request. If the Log/Logit regression model is used for results interpretation, the linearity coefficient (r^2) of the calibration curve should be no less than 0.985. An OD value of less than 0.5 absorbance units for 0ppb standard may indicate deterioration of reagents.

If a sample contains aflatoxin levels higher than the highest standard (>20 ppb), the filtered extract should be further diluted in 70% methanol such that the diluted sample results are in a range of 2.0 - 20 ppb and reanalyzed to obtain accurate results. The dilution factor must be included when the final result is calculated.

**Performance Characteristics**

**Limit of detection:** 1 ppb for corn and other commodities except for chilli which is approximately 2.5ppb and malted barley which is 1.3ppb (Determined by the average values of 10 aflatoxin-free corn samples plus 2 standard deviation)

**Limit of quantitation:** 1 ppb (Described as the lowest concentration point on the calibration curve that this test can reliably detect aflatoxin).

**Range of quantitation:** 1 – 20 ppb (For quantitation of samples above 20 ppb samples should be diluted such that the diluted sample result are in a range of 2.0 - 20 ppb).

**Materials Supplied With Kit**

- 96 antibody coated microwells (12 eight-well strips) in a microwell holder (sealed in a foil pouch)
- 96 non-coated dilution microwells (12 eight-well strips marked with blue/green at base)
- 6 vials of 1.5mL of each aflatoxin standard (0, 1.0, 2.0, 4.0, 10.0 and 20.0 ppb)
- 1 bottle of 25mL of aflatoxin conjugate (green-capped bottle)
- 1 bottle of 15mL of substrate solution (blue-capped bottle)
- 1 bottle of 15mL of stop solution (red-capped bottle)

**Materials Required But Not Provided With Kit**

**Extraction Procedure**

- *EQMMS2010: Romer Series II® Mill or equivalent*
**Assay Procedure**

- **8-channel and single channel pipettors capable of pipetting 100\(\mu\)L and 200\(\mu\)L with tips**
- ***EQOLE1300: Timer**
- ***COKAD1150: Wash bottle**
- **Distilled or de-ionized water**
- **Absorbent paper towels**
- ***3 reagent boats for use as reagent containers for an 8-channel pipettor**
- ***Microwell reader with a 450nm filter, (GIPSA approved readers: Stat Fax\textsuperscript{\textregistered} 303 Plus manufactured by Awareness Technology Inc. or the EL301 manufactured by BIO-TEK\textsuperscript{\textregistered} Instruments, Inc.) or equivalent.**

*Items available from Romer Labs, Inc.* - Americas Division

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