AgraQuant® Aflatoxin M₁
Sensitive 25/500

Order #: COKAQ7100

Intended Use

The AgraQuant® Aflatoxin M₁ Sensitive is a direct competitive enzyme-linked immunosorbent assay (ELISA) that determines a quantitative level for the presence of aflatoxin M₁ and is intended for use in milk and milk products.

The AgraQuant® Aflatoxin M₁ Sensitive has been validated for milk, milk powder and cheese.

Aflatoxin M₁

Aflatoxins are toxic and carcinogenic. They are metabolites of the fungi Aspergillus flavus and Aspergillus parasiticus. There are four principle types of aflatoxin: B₁, B₂, G₁ and G₂, which are named for their respective innate fluorescent properties. Aflatoxin B₁ is the most frequently encountered of the group and the most toxic.

Aflatoxin M₁ is a hydroxylated metabolite of aflatoxin B₁, which was first found present in milk and can be detected in urine as well. It is the most potent hepatocarcinogen known in the rat and rainbow trout. Because the young of any species is more susceptible to aflatoxin than adults, aflatoxin M₁ becomes of concern through the young children consuming milk. Processing of milk and dairy products do not lead to a significant degradation of aflatoxin M₁ and therefore can occur in such products as milk powder and cheese.

The commission of European communities set the maximum admissible level of aflatoxin M₁ in milk as 50pg/ml (ppt) in 1998. The US Food and Drug Administration action level of aflatoxin M₁ is 500pg/ml (ppt) for milk.

Assay Principles

The AgraQuant® Aflatoxin M₁ Sensitive is a direct competitive enzyme-linked immunosorbent assay (ELISA). Anti-aflatoxin M₁ antibody is coated on the surface of a microtiter wells. Aflatoxin M₁ standards or samples are mixed with enzyme-conjugated aflatoxin M₁ in the dilution wells, and then transferred to the antibody-coated microwells. Aflatoxin M₁ in standards or samples 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 M₁ 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 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. Caution must be taken in its use and storage of chemical solvents such as methanol, dichloromethane and hexane.
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 materials and containers appropriately after use.
Wash Solution Preparation
Transfer contents of Wash Solution Concentrate bottle to a 500ml plastic squeeze bottle and add 475ml distilled/deionized water. Swirl to mix.

Procedure
Sample Preparation / Extraction

Milk
1. Pipette 5ml of fresh milk sample (full-cream milk or skim milk) into a test tube and incubate for 30 minutes at 4°C.
2. Centrifuge the sample at 3000g for 10 minutes.
3. Take 0.4mL of milk serum below the fat layer and mix with 0.1mL of 100% methanol (the ratio between milk serum and methanol is 4:1).
4. The sample is now ready for ELISA test.

Milk Powder
1. Weigh 10g of skim or full-cream milk powder into a flask.
2. Dissolve milk powder with 100mL 50°C distilled or de-ionized water.
3. Homogenize it by using a magnetic stirrer.
4. Follow the above-mentioned sample preparation method for milk.

Cheese
1. Weigh 2g of cheese sample into a flask, and add 40mL of dichloromethane.
2. Extract for 30 minutes by shaking at 260rpm in rotary shaker at room temperature.
3. Take 5mL of extracts and evaporate at 60°C under a nitrogen stream or using an evaporation system.
4. Re-dissolve the residue in a mixture of 0.5mL PBS, 0.5mL methanol and 1.0mL hexane.
5. Take 0.4mL of aqueous/methanolic phase (lower part) and dilute with 0.6mL of sample diluent (dilution factor of 0.4).
6. The sample is now ready for ELISA test.

Assay
Note: All reagents and kit components must be at room temperature 18-30°C (64-86°F) before use. It is recommended that a stepper pipette be used for pipetting reagents such as Conjugate, Substrate and Stop Solution.

1. Place the appropriate number of green-bordered Dilution Strips in a microwell strip holder. One Dilution Well will be required for each standard, (i.e. 0, 25, 50, 100, 200 & 500 ppt) 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 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. Mix each well by carefully pipetting it up and down 3 times. Use a fresh pipette tip for each standard or sample. Note: Make sure the pipette tip has been completely emptied.
5. Using an 8-channel pipettor with fresh tips for each 8-well strip, transfer 100 μL of the contents from each Dilution Well into a corresponding Antibody Coated Microwell. Incubate at room temperature for 60 minutes.
6. Empty the contents of the microwell strips into a waste container. Wash by filling each microwell with Diluted Wash Buffer, 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.
7. 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.
8. 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 20 minutes under dark condition.

9. 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.

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

**Interpretation of the Results**

1. 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 six standards. Since the amount of aflatoxin M₁ in each standard is known, the unknowns can be measured by interpolation from this standard curve.

2. The dilution factors need to be used for calculations of the final aflatoxin M₁ concentration in samples:
   - For milk: 1
   - For milk powder (skim & full-cream): 10.8
   - For cheese: 8

**Performance Characteristics**

| Limit of Detection          | 18 ppt (fresh milk) |
|                            | 252 ppt (skim milk powder) |
|                            | 257 ppt (full-cream milk powder) |
|                            | 128 ppt (cheese) |

| Range of Quantitation       | 25 - 500 ppt (fresh milk) |
|                            | 270 - 5400 ppt (skim and full-cream milk powder) |
|                            | 200 - 4000 ppt (cheese) |

**Accuracy** (Recovery%, by three analysts using two different lots of test kits):

- Fresh milk: 93 - 119%
- Milk powder: 95 - 105%
- Cheese: 61 - 69%

**Precision** (CV%, by three different analysts using two different lots of test kits):

- Fresh milk at 50ppt: 8.5%
- Skim milk powder at 2160ppt: 6.1%
- Cheese at 800ppt: 6.1%

**Cross reactivity relative to Aflatoxin M₁ (100%):**

- Aflatoxin B₁: 88%
- Aflatoxin B₂: 27%
- Aflatoxin G₁: 11.5%
- Aflatoxin G₂: 4.7%

**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 bordered with green at well top)
- 6 vials of 1.5mL of each aflatoxin M₁ standard (0, 25, 50, 100, 200 and 500 pg/ml), ready-to-use.
- 1 bottle of 25mL of aflatoxin conjugate, ready-to-use.
- 1 bottle of 15mL of substrate solution, ready-to-use.
- 1 bottle of 15mL of stop solution, ready-to-use.
- 1 bottle of 10mL of sample diluent, ready-to-use.
Materials Required But Not Provided With Kit

Extraction Procedure
- *EQOLE1010: Balance, 400 g
- *EQOLE1050: Graduated cylinder: 100mL
- Volumetric flasks with stopper
- Horizontal shaker or magnetic stirrer
- Vortex
- Centrifuge
- *Evaporation system
- Vials for evaporation: 20-30mL
- Solvent: ACS grade methanol, dichloromethane and hexane
- Distilled or de-ionized water

Assay Procedure
- Multi-pipettor
- Single channel pipettors capable of pipetting 50, 100, 500 and 1000μL 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 reading filter and a 630nm differential filter

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

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