Aflatoxin M1 ELISA KIT
For the Quantitative analysis of Aflatoxin M1 in milk, milk powder and cheese
Code EEM005096 Format 96 tests

1 - INTENDED USE

Aflatoxins are toxic metabolites produced by a variety of molds such as Aspergillus flavus and Aspergillus parasiticus. They are carcinogenics and can be present in grains, nuts, cottonseed and other commodities associated with human food or animal feeds. Crops may be contaminated by one or more of the four following sub-types of aflatoxin: B1, B2, G1 and G2. Aflatoxin B1 is the most toxic and frequently detected form. The other types present a significant danger if the concentration is at a high level. Aflatoxin have been implicated in human health disorders including hepatocellular carcinoma, aflatoxicosis, Reye's syndrome and cronic hepatitis. Animals are exposed to aflatoxins by consumption of feeds that are contaminated by aflatoxin producing fungal strains during growth, harvest or storage. When cows are fed contaminated feed, aflatoxin B1 is converted by hydroxylation to aflatoxin M1, which is subsequently secreted in the milk of lactating cows. Aflatoxin M1 is quite stable towards the normal milk processing method such as pasteurization and if present in raw milk, it may persist into final products for human consumption. Most controlling government agencies worldwide have regulations regarding the amount of aflatoxin M1 in milk and milk products. In the EU the limit for the presence of M1 in milk and reconstituted milk powers has been set at 50 ppt.
EuroClone AFLATOXIN M1 ELISA KIT is a competitive enzyme immunoassay for the quantitative detection of Aflatoxin M1 in milk, milk powder and cheese.

2 - TEST PERFORMANCES

Matrices
Milk (raw and homogenized) , milk powder and cheese

Sample preparation
only for cheese;
No milk sample preparation is required (fast centrifugation only for raw milk)

Incubation Time
30 minutes + 15 minutes + 10 minutes
Quantification range 5ppt - 100ppt

Precision
Intrassay CV<10%

Interassay CV<20%

3 - PRINCIPLE OF THE ASSAY

The assay is performed in ELISA microwells coated with a high affinity anti-Aflatoxin M1 antibodies. Standard and samples are added into the microwells and the Aflatoxin M1 contained in the standards or in the samples, are bound by the coated antibodies. After allowing this reaction to proceed, the unbound material is removed in a washing step. The Aflatoxin M1-Peroxidase conjugate, that binds to the antibody binding sites not already occupied by aflatoxin M1, is added during a second reaction. After a short incubation time a further washing step is performed. The bound enzyme activity is determined by adding a fixed amount of chromogenic substrate. Bound Enzyme Conjugate converts the colourless substrate into a blue product. The substrate reaction is stopped by the addiction of sulphuric acid, which leads to a colour change from blue to yellow. Absorbance is then measured spectrophotometrically (450 nm) and colour intensity results are inversely proportional to the original Aflatoxin M1 concentration in the sample. Sample concentrations are then calculated on the basis of a calibration curve derived from standards of known Aflatoxin M1 concentration.

4 - KIT CONTENT

• 1 x Anti- Aflatoxin M1 Coated Microplate (1 x 96 wells, 12 strips, breakable wells)
• 6 x Aflatoxin M1 Standard Solutions (3ml/each): 0 pg/ml, 5 pg/ml, 10 pg/ml, 25 pg/ml, 50 pg/ml, 100 pg/ml
• 1 x Aflatoxin M1 Conjugate (22 ml), red cap
• 1 x Washing Buffer 10X (50 ml)
• 1 x Chromogen Solution (22 ml), brown bottle
• 1 x Stop Solution (6 ml), white cap
• 1 x Sample Buffer (50 ml)
• 1 x Box insert.

5 - MATERIALS REQUIRED BUT NOT PROVIDED

• Vortex mixer
• Balance
• 10-200 µl precision micropipette with suitable tips
• 50-200 µl multichannel micropipette with suitable tips
• Reservoir (disposable) for multichannel pipette
• Squeeze bottle or microplate washer
• Microtiter plate reader equipped with 450 nm and 620 nm filters. The use of a calibrated ELISA Reader Equipment able to detect Optical Density in the range of 0.05-3.0 is required
• Microcentrifuge
• Distilled water
• 10 ml pipette and pipette-holder
• Test tube racks

• Graduated Cylinders
• Timer (range 60 min.)
• Disposable gloves
• Absorbent paper towels
• Glass Tube
• Grinder or similar
• Methanol (analytical grade)

6 - PREPARATION OF WORKING SOLUTION

• Aflatoxin M1 Standard Solutions: ready-to-use.
• Aflatoxin M1 Conjugate: ready-to-use.
• Washing Buffer: dilute the Washing Buffer 10X Concentrated 1:10 using distilled water (i.e. 10ml + 90ml). Concentrated
salt solution may form precipitates when stored at 2-8℃. This is not affecting the reagent's performance. In case of precipitates, bring the buffer to room temperature and mix well to bring salts back into solution. Prepare only the amount
required and discard left washing buffer diluted at the end of the assay.
• Chromogen Solution: ready-to-use. Chromogen and substrate solution are light sensitive, therefore, avoid exposure to direct light.
• Stop Solution: ready-to-use. Contains sulphuric acid: handle with care and in case of contact, wash thoroughly with tap water.
• Sample Buffer: ready-to-use.

7 - SAMPLE PREPARATION

<u>Raw Milk</u>
• An aliquot of unprocessed raw fatty milk should be placed at refrigerated temperature overnight to allow the fat globules
to rise the surface in a natural "creaming" effect. Centrifugation at this point is not necessary.
• Alternatively, if the sample is at ambient temperature or has been mixed in transit, place an aliquot at refrigerated
temperature for 1-2 hours and centrifuge at 2000 x g for 5 minutes to induce separation of upper fatty layer.
• Remove the upper fatty layer by aspiration and use the lower plasma in the assay.
Homogenized Milk
• Homogenized skim milk should be used directly in the assay.
• Due to the stabilization of the fat globules induced by the homogenizing process they are difficult to eliminate even by
high speed centrifugation to create plasma from homogenized fatty milk. Therefore use homogenized fatty milk directly in
the assay.
<u>Milk Powder</u>
• Reconstitute milk powders according to the manufacturer's instructions and treat the reconstituted product as above.
<u>Cheese</u>
• Grind the samples.
• Weight 1 g of grinded sample and add 5 ml of Methanol in a glass tube.
• Vortex for 5 minutes.
• Centrifuge for 5 minutes at 5000 x g .
• Transfert1 ml of the clean supernatant in a glass tube and evaporate.
• Suspend the evaporated sample with 1 ml of sample buffer and vortex for 1 minutes, leave for 5 min at room temperature

and vortex for 1min.
• Centrifuge for 5 minutes at 5000 x g.
• Dilute 1:2 (0,3 ml of clear supernatant with 0,3 ml ofsample buffer).
• Load the sample into the plate

8 - Precautions
• Always wear disposable gloves when using the device.
• In case of ingestion or contact with the eyes, skin or mucosae, wash with plenty of water and consult a physician.
• If all the kit is not to be used at one time, remove only the number of wells needed for the day's testing and place the remaining strips in foil pouch with desiccant at 2-8℃.
• Take only the appropriate amount of the reagents provided, leaving the bulk in the original vial.
• For reagents to be dispensed by Multichannel pipette, increase the volume by 1 ml.
• Bring all reagents to room temperature (20-25℃) before use.
• Once started, complete all assay steps without interruption.
• Adhere to all time and temperature conditions stated in the procedures
• Note: the reproducibility of enzyme immunoassay depends on the uniform washing of the wells. Improper washing leads
to high background, poor precision and low signal. Carefully follow the described washing sequence for a precise test.
• Please note: if you don't have a microtiter plate reader that allows OD>2 units the Maximum Binding values could be in OVERFLOW.

8-A - Recommended assay layout
• Predispose a duplicate for the Maximum Binding (standard zero: i.e. water) and a duplicate for the Blank.
• Predispose a duplicate for each standard curve point and a duplicate for each sample.

8-B - Assay procedures
According to the number of samples, including the controls, to be tested, remove the required number of wells. Place the
remaining wells in the pouch with the desiccant gel and reseal the pouch. For short time storage, the remaining wells can be stored in the plastic envelope supplied always with desiccant gel.
Use a single disposable tip for each pipetting step to avoid cross contamination. Do not allow tips to touch the liquid already in the microwells or the inner well surfaces.
1. Place 200 µl of distilled water into the Blank wells in duplicate.
2. Place 200 µl of standards and samples into the appropriate wells in duplicate.
3. Mix gently by rocking the plate manually.
4. Incubate 30 minutes at room temperature (20-25℃), preferably away from direct light.
5. Discard the content of the wells into a appropriate receptacle. Fill completely all the wells with diluted washing buffer (approx. 300 µl/well); empty wells by inverting the plate; repeat the washing step 2 times (for a total of 3 times). Finally, tap vigorously the microwell holder upside down against absorbent laboratory paper to ensure complete liquid removal.
6. Add 200 µl of Enzyme Conjugate (red cap) to each well except the Blank wells.
7. Mix gently by rocking the plate manually.
8. Incubate 15 minutes at room temperature (20-25℃), preferably away from direct light.
9. Repeat step 5 (washing step for a total of 3 times)
10. Using the multichannel micropipette, add 200 µl of Chromogen Solution to each well, including the Blank wells.
11. Incubate 10 minutes at room temperature, in the dark.

12. Stop the reaction by adding 50 µl/well Stop Solution. Colour turns from blue to yellow. Mix gently by rocking the plate manually.
13. Read the optical density (OD) of each microwell with a microplate reader at 450 nm.

9 – CALCULATION

The unknown values for Aflatoxin M1 concentration in samples are determined from a calibration curve.

• Calculate the mean absorbance value for Blank and subtract it from the mean of absorbance values.

• Calculate the mean absorbance value for the Maximum Binding, the standards and the samples.

• Divide the mean absorbance value of standards and samples (B) by the mean absorbance value of the Maximum Binding (Bo) and multiply by 100. Maximum binding is thus made equal to 100% and the absorbance values are quoted in percentages:

$$\frac{\text{Absorbance standard (or sample)}}{\text{Absorbance Maximum Binding}} \times 100 = \frac{B}{Bo} (\%)$$

• Enter the B/Bo (%) values calculated for each standard in a semi-logarithmic system of coordinates against the Aflatoxin
M1 standard concentration (pg/ml); draw the standard curve.

• Take the B/Bo (%) value for each sample and interpolate the corresponding concentration from the calibration curve.

10 - RESULTS

The Aflatoxin M1 concentration in ppt (pg/ml) corresponding to the absorbance of each sample can be read from the calibration curve.
For cheese samples the dilution factor is 10.
In the EU the limit for the presence of Aflatoxin M1 in milk and reconstituted milk powders has been set at 50ppt.
The recovery of 50 ppt spiked into milk is 80-90%.
The recovery for cheese is 70-80%.

11 – NOTES, STABILITY AND STORAGE

• The kits should be stored at 2-8℃ and used before the expiry date printed on the label on the box.

• Do not freeze.

• The standard curve should be discarded after use.

• Diluted Washing Buffer should not be stored for more than 8 hours and must be discarded after use.

• Once open, if properly stored (resealed in dry atmosphere at 2-8℃), the microwell plate can be maintained for several months.

• Do not interchange individual reagents between kits of different lot numbers.

• Do not dilute reagents as this will result in loss of sensitivity and poor performance.

• Do not return unused reagents back into their original bottles. The assay procedures details volumes required.

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