l'screen AFLA M1

Enzyme immunoassay for the detection of Aflatoxin M_1 (Cat. nr. HU0040002)

l'screen **AFLA M1** is a kit prepared for a competitive immunoenzymatic assay for the quantitative analysis of aflatoxin M_1.
The kit contains the procedure and the materials sufficient for 96 determinations including standards.
For result evaluation a microtiter plate or a strip photometer is required (manual or automatic ELISA reader).

Type of samples that can be analyzed (matrices)
Milk (raw bovine, sheep, goat, buffalo milk; whole, partially skim and skim bovine UHT, powdered bovine milk), bovine cheese, sour cream, yoghurt, mozzarella cheese.

Sample preparation
- Raw milk: refrigeration at +2/+8°C, centrifugation.
- UHT milk: direct analysis or refrigeration at +2/+8°C and centrifugation (optional)
- Powdered milk: dilution.
- Cheese, sour cream: extraction with solvent, filtration, evaporation, reconstitution, centrifugation, dilution.
- Firm cheese (alternative method): protease digestion, incubation, centrifugation, filtration, neutralization, dilution.
- Yoghurt, mozzarella cheese: extraction with solvent, centrifugation, evaporation, reconstitution.

Assay time: 75 minutes (sample preparation not included).

Detection limit
- Raw milk, UHT milk: 5 ng/l
- Powdered milk: 50 ng/l
- Cheese, sour cream: 37 ng/Kg
- Firm cheese (alternative method): 120 ng/Kg
- Yoghurt, mozzarella cheese: 25 ng/Kg

Specificity

Compound	Cross-reactivity (%)
Aflatoxin M_1	100
Aflatoxin M_2	16
Aflatoxin B_1	< 0.1
Aflatoxin B_2	< 0.1
Aflatoxin G_1	< 0.1
Aflatoxin G_2	< 0.1

1. TEST PRINCIPLE

The assay is performed in plastic microwells which have been coated with anti-Aflatoxin M_1 antibodies. Aflatoxin M_1 standard solutions and samples are added to the microwells.
During the first incubation, free Aflatoxin M_1 molecules are bound to the anti-Aflatoxin M_1 antibodies.
Any unbound substance is then removed in a washing step.
A second incubation is performed with an aflatoxin-HRP conjugate, which covers all the remaining free binding sites of the antibody. After the incubation a second washing step is performed.
The bound enzyme activity is determined by adding a fixed amount of a chromogenic substrate.
The enzyme converts the colorless chromogen into a blue product during the third incubation.
The addition of the stop reagent leads to a color change from blue to yellow. The absorbance is measured by a microplate reader at 450 nm. The color development is inversely proportional to the Aflatoxin M_1 concentration in the sample.

2. PROVIDED REAGENTS

Microtiter plate: 96 wells (12 strips x 8 wells) coated with anti-Aflatoxin M_1 antibodies.
As the strips are breakable, the wells can be used individually. For this purpose, it is sufficient to take out the wells from the sheath and to break the joint.
1 Cover for covering the microtiter plate or strips during incubation.
Aflatoxin M_1 standard solutions: 7 plastic vials containing 1.5 ml of: 0 ng/l; 5 ng/l; 10 ng/l; 25 ng/l; 50 ng/l; 100 ng/l; 250 ng/l.
Enzyme conjugate:1 plastic bottle containing 14 ml.
Milk Diluent: 1 plastic bottle containing 12 ml.
Extraction buffer: 2 plastic bottles containing 30 ml.
Washing-buffer 20X: 1 plastic bottle containing 50 ml.
Developing solution:1 plastic bottle containing 14 ml.
Stop solution: 1 glass bottle containing 15 ml. White cap.

3. MATERIALS REQUIRED BUT NOT PROVIDED

For sample preparation:

	Raw milk	Cheese, sour cream	Cheese Firm	Yoghurt, mozzarella cheese
Centrifuge*	x	x	x	x
Plastic tubes	x	x		x
50 ml centrifuge tubes			x	
Incubator with shaker			x	
Cellulose filter			x	
Pepsin (Sigma;cod. P-7000)			x	
0,1N HCl			x	
pH meter			x	
0,5N NaOH			x	
Glass tubes with screw cap		x		
Evaporator		x		x
Balance		x	x	x
Vortex		x		x
Shaker		x		x
Hexane		x		
Dichloromethane		x		
Methanol				x
Chemical hood		x		x
Glass pipettes		x		
Micropipette 100-1000 µl, tips		x		x

* preferably refrigerated

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For assay setting

- Microplate reader, filter 450 nm.
- Micropipette 50-200 µl, tips.
- Multichannel micropipette 50-250 µl, if using more than three strips.

4. WARNING AND PRECAUTIONS FOR THE USERS

- The test is for *in vitro* diagnostic use only.
- Some reagents contain solutions that may be identified as dangerous substance by the Regulation (EC) *N° 1272/2008.* Please refer to *Material Safety Data Sheet* available on both the Eurofins Technologies and Eurofins Tecna (*tecna.eurofins-technologies.com)* web site.
- Handle the reagents with caution, avoiding contact with skin, eyes and mucous membranes.

5. HANDLING AND STORAGE INSTRUCTIONS

- Store the kit at +2/+8 °C and never freeze.
- **Bring all reagents to room temperature before use (2 hours). ATTENTION: Do not unseal the microplate until it reaches the room temperature.**
- Reseal the unused strips of the microtiter plate in the bag together with the desiccant bag provided.
- Return all reagents to +2/+8 °C immediately after use.
- Do not use components after the expiration date.
- Do not intermix components from different kit lots.
- Do not use photocopies of the instruction booklet. Follow the original instruction booklet that is included with the kit.
- Do not change the assay procedure, in particular:
  - do not prolong the incubation times;
  - do not incubate the plate at temperatures higher than 25°C;
  - do not shake the plate during the incubations.
- Use accurate and precise micropipettes with suitable tips for dispensing.
- Once started, complete all the steps without interruption.
- The reproducibility of ELISA results depends largely upon the efficiency and uniformity of microwells washing; always keep to the described procedure.
- Use a single disposable tip for each standard solution and sample to avoid cross-contamination.
- Do not allow tips to contact the liquid already in the microwells.
- Avoid exposure to direct light during all incubations. It is recommended to cover the microtiter plate without using plate sealers.

6. SAMPLES PREPARATION

6.1. Raw milk

After milking, the milk has to be tested within 24 hours. Otherwise milk has to be stabilized (with sodium azide, azidiol or similar substances)

1) Refrigerate the sample and centrifuge it at +2/+8°C for 10 minutes at 3000xg.
2) Separate the fat from the skimmed milk.
3) Use the skimmed milk directly in the assay, after it is adjusted to room temperature.
4) In the application of the 25 – 1250 ng/l measuring range, dilute the samples with the milk diluent 5x (100µl of the sample + 400 µl of milk diluent); to obtain the effective aflatoxin M1 concentration in samples, the concentration read from the calibration curve must be multiplied by 5.

6.2. Raw milk

As an alternative option to procedure 6.1, it is possible to analyze raw milk without skimming.

After milking, the milk has to be tested within 24 hours. Otherwise milk has to be stabilized (with sodium azide, azidiol or similar substances).

Use the milk directly in the assay, after it is adjusted to room temperature.

6.3. UHT bovine milk (whole, partially skim, skim)

Use the milk directly in the assay.

Alternative procedure

1) Refrigerate the sample and centrifuge it at +2/+8°C for 10 minutes at 3000xg.
2) Separate the fat from the skim milk.
3) Use the skim milk directly in the assay, after it is adjusted to room temperature.

6.4. Powdered bovine milk

1) Weigh out 10 g of the powder and get to a volume of 100 ml with distilled water.
2) Shake until the powder is completely dissolved.
3) The dilution factor is 10.

6.5. Cow's milk cheese, sour cream

1) Weigh 2 g of sample in a screw cap glass test tube.
2) Add 15 ml of dichloromethane and extract by shaking the vial for 15 min.
3) Filter the suspension.
4) Transfer 3.75 ml of the extract into a glass tube and evaporate at 60 °C under a slow air or nitrogen stream.
5) Re-dissolve the residue in 750 µl of extraction buffer and mix thoroughly for 1 min (vortex).
6) Add 750 µl of hexane, extract by vortex for 1 min.
7) Centrifuge for 15 min at 2000xg.
8) Remove the hexane upper layer.
9) Take a 50 µl aliquot of the methanolic/aqueous phase and dilute it in a small tube (1.5 ml) with 200 µl of milk diluent. Mix gently.
10) The dilution factor is 7.5.

6.6. Alternative procedure of firm cheese

1) Finely grind the cheese sample.
2) Weigh 3 g of the sample in a 50 ml centrifuge tube.
3) Add 30 ml of a digestion solution of 0.2% pepsin in 0.1N HCl.
4) Shake for 16 hours in an incubator at 42°C.
5) Centrifuge for 15 minutes at 4500xg at room temperature.
6) Filter the supernatant using a paper filter in order to remove the fat fraction.
7) Recover 10 ml of the filtered solution and neutralize it with 2 ml of 0.5N NaOH. Make sure pH is between 7 and 7.5.
8) Dilute the sample in ratio 1:1 with the milk diluent (ex: 200 µl + 200 µl).
9) The dilution factor is 24.

6.7. Yoghurt, mozzarella cheese

1) Weigh 1 g of sample and add 5 ml of methanol. For mozzarella cheese, mince the sample before adding the solvent.
2) Shake thoroughly for 5 min.
3) Centrifuge for 5 min at 5000xg.
4) Transfer 0.25 ml of the upper organic phase and evaporate at 40 °C under a slow air or nitrogen stream.
5) Dissolve the residue in 0.25 ml of milk diluent and mix thoroughly for 1 min (vortex); leave it to stand for 5 minutes.
6) The dilution factor is 5.

7. WORKING SOLUTIONS PREPARATION

Aflatoxin M1 standard solutions: ready to use **(shake gently prior to use).**
Enzyme conjugate: ready to use.
Milk Diluent: ready to use.
Extraction buffer: ready to use.
Washing buffer: dilute the concentrate 1:20 (1+19) with distilled water. **ATTENTION:** If crystals are present, bring the solution to room temperature and stir in order to solve them completely.
The diluted washing buffer is stable at room temperature for 24 hours and at +2/+8°C for two weeks.
Developing solution: ready to use. The solution is light sensitive and must be stored away from direct light.
Stop solution: ready to use. **CAUTION:** It contains 1M sulphuric acid. Handle with care; in case of contact flush immediately with plenty of water.

8. ASSAY PROCEDURE

1) Predispose an assay layout, recording standard solutions and samples positions, taking into account that all have to be run in duplicate.
2) First incubation
   - Add 100 µl of each standard/ sample into the corresponding wells.
   - Shake the plate gently with rotatory motion for few seconds and cover it with the cover.
   - Incubate 45 minutes at room temperature.
   - Do not prolong the first incubation time and do not use automatic shakers.
3) Washing
   - Pour the liquid out from the wells.
   - Fill completely all the wells with working wash solution using a squeeze bottle. Pour the liquid out from the wells.
   - Repeat the washing sequence four (4) times.
   - Remove the remaining droplets by tapping the microplate upside down vigorously against absorbent paper.

Do not allow the wells to dry out.

4) Second incubation
   - Using a multichannel pipet, add to the wells 100 µl of the enzyme conjugate solution.
   - Shake the plate gently with rotatory motion for few seconds and cover it with the cover.
   - Incubate for 15 minutes.
5) Repeat step 3.
6) Developing
   - Using the multichannel micropipette, add 100 µl of developing solution to each well.
   - Mix thoroughly with rotatory motion for few seconds and cover it with the cover.
   - Incubate for 15 minutes at room temperature.
7) Using a multichannel pipet, add 50 µl, alternatively 100 µl of stop solution to each well and mix thoroughly with rotatory motion for few seconds.
8) Measure the absorbance at 450 nm.
9) Read within 60 minutes.
10) In case a strip reader is used, it is necessary to take out the strip from the frame and to remove the case round the wells.

9. CALCULATION OF RESULTS

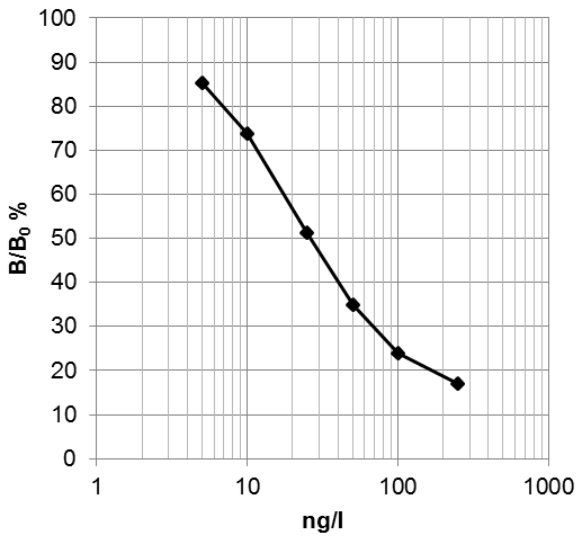
- Calculate the mean absorbance of each control, standard and sample.
- Divide the mean absorbance value of each standard and sample by the mean absorbance of the standard 0 and multiply by 100; the standard 0 is thus made equal to 100% and all the other absorbance values are expressed as percentages:

$$\frac{\text{Standard (or sample) absorbance}}{\text{Standard 0 } (B_0) \text{ absorbance}} \times 100 = \frac{B}{B_0}(\%)$$

- Enter the B/ B_0 values calculated for each standard in a semi-logarithmic system of coordinates and draw the standard curve.
- Interpolate the B/ B_0 value of each sample to the corresponding concentration from the calibration curve. For dilution applications multiply this concentration for the dilution factor, as reported in chapter 6.

Please note: For results calculation, Excel spreadsheets are available on Eurofins Tecna website tecna.eurofins-technologies.com and can be downloaded directly from the bottom of the product page.

10. CALIBRATION CURVE EXAMPLE



11. EVALUATION OF RESULTS

After processing the results, it is necessary to verify the assay performance. The verification is performed by comparison of obtained data with those given in kit specifications (see chapter 12).
If the values are outside the specifications given, then the results of the test are not assured, therefore the aflatoxin M_1 concentration levels in the samples may not be valid.
In these cases, it is advised to check the expiry date of the kit, the wavelength at which the reading was performed, as well as the procedure followed.
If operation errors are not identified as cause, contact our technical assistance.

WARNING: Kit replacement will only be possible in case of return. The kit must be stored in its integral version at +2/+8°C.

12. KIT SPECIFICATIONS

12.1. Assay specification

Description	Specifications
Mean Bo absorbance	≥ 0.7 OD_{450nm}
B/ B_0 50 %	18 - 50 ng/l
Std duplicates mean C.V.	≤ 6 %

12.2. Assay performance

Raw bovine milk	
LOQ	5 ng/l
Recovery (satisfactory range) for spiked* samples	80 -120 %
Recovery (satisfactory range) for incurred* samples	80 -140 %

* concentration: between 30 and 60 ng/l of aflatoxin M1

The results were obtained by means of a "4 parameters" elaboration of the calibration curve.

Notes: *This is considered to be a screening method; before a legal action, samples detected as positives (according to the EU law concentration higher than 0.05 µg/Kg) must be confirmed by HPLC.*

13. LIABILITY

Samples evaluated as positive using the kit have to be re-tested with a confirmation method.
Eurofins Technologies Hungary shall not be liable for any damages to the customer caused by the improper use of the kit and for any action undertaken as a consequence of results.
Eurofins Technologies Hungary shall not be liable for the unsafe use of the kit out of the current European safety regulations.

14. LITERATURE

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