Enzyme immunoassay for the detection of Aflatoxin M_1 in milk

(code MA440/MA441)

I'screen AFLA M_1 milk is a kit prepared for an immunoenzymatic assay for the quantitative analysis of aflatoxin M_1.
The kit contains the procedure and the materials sufficient for 96 determinations ***(code MA440)*** or 48 determinations ***(code MA441)*** including standards.
A microtiter plate photometer or a strip photometer is required.

Analysable samples
Raw milk, milk powder.

Sample preparation
- Raw milk: refrigeration at +2/+8°C, centrifugation.
- Milk powder: diluition.

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

Detection limit
- Raw milk,: 0,005 ppb
- Milk powder: 0,05 ppb

Specificity

Compound	Cross-reactivity (%)
Aflatoxin M_1	100
Aflatoxin M_2	16

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. The bound enzyme activity is determined by adding a fixed amount of a chromogenic substrate. The enzyme converts the colourless 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 colour development is inversely proportional to the Aflatoxin M_1 concentration in the sample.

2 PROVIDED REAGENTS

Microtiter plate: coated with anti-Aflatoxin M_1 antibodies.
CodeMA440: 96 wells (12 strips x 8 wells).
CodeMA441: 48 wells (6 strips x 8 wells).
As the strips are breakable, the wells can be used individually. For this purpose, it is sufficient to get 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: 7 amber 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 vial containing 250 µl of enzyme conjugate concentrate.
Enzyme conjugate diluent: 1 plastic vial Red cap. Code MA440: containing 20 ml; code MA441: containing 10 ml.
Washing-buffer 20X: 1 plastic bottle containing 50 ml.
Developing solution: 1 plastica bottle containing 15 ml.
Stop solution: 1 glass vial containing 9 ml. White cap.

3 MATERIALS REQUIRED BUT NOT PROVIDED

- Distilled water

Equipment
- Centrifuge, preferably a refrigerated centrifuge, in particulary if the centrifuge is a low speed;
- Plastic tubes.
- Microplate reader, filter 450nm;
- Micropipette 50-200 µl, tips;

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- Multichannel micropipette 50-250 µl *

* If using a limited number of wells (approximately not more than three strips), the multichannel micropipette is not necessary.

4 WARNING AND PRECAUTIONS FOR THE USERS

- For *in vitro* diagnostic use only.
- Some reagents contain preservative. The stop solution contains sulphuric acid and is corrosive. The enzyme conjugate is harmful.
- Handle the reagents with extreme caution, avoiding contact with skin, eyes and mucous membranes.
- Safety data sheet available on Tecna's web site.

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 dessicant bag provided
- Return all reagents to +2/+8 °C immediately after use.
- Do not use components after the expiration date.
- Do not intermix components between different kit lots.
- Do not use fotocopies of the instruction booklet. Keep always to the instruction booklet which is included inside 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 or the internal microwells surface.
- 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 stabilised (with azidiol or similar substances)

- Refrigerate the sample and centrifuge it at +2/+8°C for 10 minutes at 3000xg.
- Separate the fat from the skimmed milk.
- Use the skimmed milk directly in the assay.
- In the application of the 10 – 500 ppt measuring range, dilute the samples with the sample diluent 2x (code MA444; 100µl of the sample + 100µl of sample diluent); to obtain the effective aflatoxin M1 concentration in samples, the concentration read from the calibration curve must be multiplied by 2.
- In the application of the 25 – 1250 ppt measuring range, dilute the samples with the sample diluent 5x (code MA444; 100µl of the sample + 400µl of sample diluent); to obtain the effective aflatoxin M1 concentration in samples, the concentration read from the calibration curve must be multiplied by 5.
- In the application of the 50 – 2500 ppt measuring range, dilute the samples with the sample diluent 10x (code MA444; 50µl of the sample + 450µl of sample diluent); to obtain the effective aflatoxin M1 concentration in samples, the concentration read from the calibration curve must be multiplied by 10.

6.2 Powdered milk

- Weight out 10 g of the powder and get to a volume of 100 ml with distilled water.
- Shake until the powder is completely dissolved. Proceed as above. The dilution factor is 10.

7 WORKING SOLUTIONS PREPARATION

Aflatoxin M_1 standard solutions: ready to use
(shake gently prior to use).
Enzyme conjugate: ATTENTION: in order to recover the total coniugate amount, before use, centrifuge the vial briefly at low speed (spin-dow).
Calculate and prepare the quantity necessary for the experiment. Dilute the conjugate **1/100** with the enzyme diluent (for example, 20 µl of enzyme conjugate concentrate + 1980 µl of enzyme conjugate diluent).
Mix gently by inverting the vial. DO NOT VORTEX.
Do not take the Enzyme Conjugate concentrate in volume lower of 20 µl.
Washing buffer: dilute the concentrate 1:20 (1+19) with distilled water. ATTENTION: in presence of crystals, bring the solution at 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.
Stop solution: ready to use.
Enzyme conjugate diluent: ready to use.

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8 ASSAY PROCEDURE

Wait until all kit components reach room temperature before use (2 hours).

1. Predispose an assay layout, recording Maximum Binding (Bo or standard 0), standard solutions and samples positions, taking into account that all have to be run in duplicate.

Remove the strips not to be used from the frame and replace them in the pouch with the dessicant. Reseal the pouch tightly.

First incubation

2. Add 100 µl of each standard/ sample into the standard wells.

Shake the plate gently with rotatory motion for few seconds and cover it with the cover.

3. Incubate 45 minutes at room temperature;

Do not prolong the first incubation time and do not use automatic shakers.

4. 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.
- Remove the remaining droplets by tapping the microplate upside down vigorously against absorbent paper;
- Repeat the washing sequence four (4) times.

Do not allow the wells to dry out

Second incubation

5. 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.

6. Incubate for 15 minutes.

7. Repeate step 4.

Developing

8. 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.

9. Incubate for 15 minutes at room temperature.

10. Using a multichannel pipet, add 50 µl of stop solution to each well and mix thoroughly with rotatory motion for few seconds.

11. Measure the absorbance at 450 nm.

Read within 60 minutes.

In case an 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

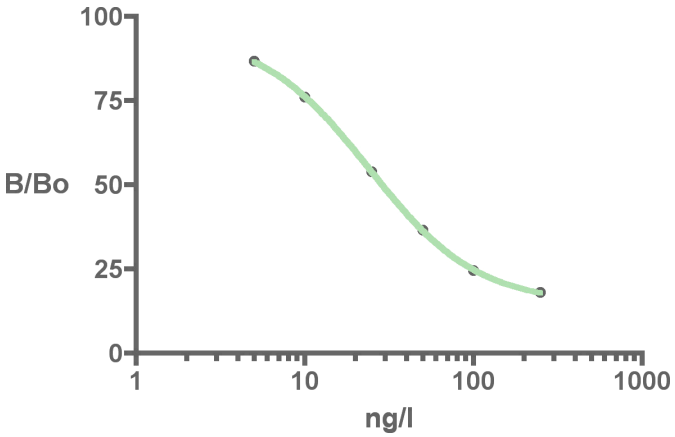
- Calculate the mean absorbance of each controls, standards and samples.
- Divide the mean absorbance value of each standard and sample by the mean absorbance of the standard 0 and multiply by 100; the standard 0 is thus made equal to 100% and all the other absorbance values are expressed as percentages:

$$\frac{\text{absorbance of standard (or sample)}}{\text{absorbance of standard 0 } (B_o)} \times 100 = \frac{B}{B_o} \ (\%)$$

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

For calculation of the ELISA results, Excel spreadsheets can be downloaded from the section "download" in Tecna's web site, www.tecnalab.com.

10 EXAMPLE OF CALIBRATION CURVE



11 RESULTS EVALUATION

After results elaboration, it is necessary to verify the assay performance. The verification is performed by comparision of obtained data with those given in kit specifications. If the values are out from the specifications given, it is advised to control the expiry date of the kit, the wavelenght of absorbance recordering, as well as the procedure employed. If operation errors do not emerge, contact our technical assistance.

WARNING: substitution will be possible just in case of rendered kit. The kit must be conserved in its integral version and at the temperature indicated in this booklet.

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12 KIT SPECIFICATIONS

12.1 Assay specification

Description	Specifications
Mean Bo absorbance	≥ 0,7 OD_{450nm}
B/Bo 50 %	20 - 50 ng/l (ppt)
Std duplicates mean C.V.	≤ 6 %

12.2 Assay performance

Raw Milk	
LOQ	5 ppt
Mean recovery (+/- std.dev.) at 50 ppt*	110 (± 15) %

* spiked samples (*n*=10)

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 50 ppt) must be confirmed by HPLC.

13 BIBILIOGRAPHY

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14 LIABILITY

Samples evaluated as positive using the kit have to be re-tested with a confirmation method.
Tecna 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.
Tecna shall not be liable for the unsafe use of the kit out of the current European safety regulations.

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