INSTRUCTION FOR USE

I'screen AFLA M_1 Milk

(Cat. nr. HU0040001)

Enzyme immunoassay for the detection of Aflatoxin M_1 in milk

PERFORMANCE TESTED AOAC RESEARCH INSTITUTE LICENSE NUMBER 072002

I'screen **AFLA M_1 Milk** 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 the result evaluation a microtiter plates or a strips photometer is required (manual or automatic ELISA reader).

Intended use
I'screen **AFLA M_1 Milk** is intended for the quantitative analysis of aflatoxin M_1 in raw whole milk, raw skim milk and powdered milk.

Sample preparation
- Raw bovine milk: refrigeration at +2/+8°C, centrifugation.
- Powdered bovine milk: dilution.

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

Detection limit
- Raw bovine milk: 5 ng/L
- Powdered bovine milk: 50 ng/L

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

Cross-reactivity was calculated as ratio of B/B_0 values corresponding to 50% inhibition (AFM_1 B/B_0 50% / compound B/B_0 50%).

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 of 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 get out the wells from the sheath and to break the joint.
1 plastic plate cover to protect the microtiter plate or strips during incubation.
Aflatoxin M_1 standard: 7 plastic vials containing 1.5 ml Aflatoxin M_1 solution in the following concentrations: 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.
Washing-buffer 20X: 1 plastic bottle containing 50 ml.
Developing solution: 1 plastic bottle containing 14 ml.
Stop solution: 1 glass vial containing 8 ml. White cap**.**

3. MATERIALS REQUIRED BUT NOT PROVIDED

- Distilled water

Equipment
- Centrifuge, preferably refrigerated
- Plastic tubes
- Microplate reader, filter 450nm
- Micropipette 50-200 µL, tips
- Multichannel micropipette 50-250 µL, if using more than three strips
- Milk diluent (dilution buffer for milk samples, 2x12 mL), cat.nr. HU0040101

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4. WARNING AND PRECAUTIONS FOR THE USERS

- The test for in vitro diagnostic use only.
- Some reagents contain solutions that may be identified as dangerous substance by the Regulation (EC) No 1272/2008. Please refer to Material Safety Data Sheet available on Gold Standard Diagnostics web site: *www.goldstandarddiagnostics.com.*
- 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 change 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 with the plastic plate cover. Do not use plate sealers.

6. SAMPLES PREPARATION

6.1. Raw bovine 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). Guidelines for proper milk sampling for analysis can be found in EU Regulation (EC) 401/2006.

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 for application of the 5 – 250 ng/l measuring range.
4) In the application of the 10 – 500 ng/L measuring range, dilute the samples with the sample diluent 2x (cat.nr. HU0040101; 100µL of the sample + 100µL of sample diluent); to obtain the effective aflatoxin M_1 concentration in samples, the concentration read from the calibration curve must be multiplied by 2.
5) In the application of the 25 – 1250 ng/L measuring range, dilute the samples with the sample diluent 5x (cat.nr. HU0040101; 100µL of the sample + 400µL of sample diluent); to obtain the effective aflatoxin M_1 concentration in samples, the concentration read from the calibration curve must be multiplied by 5.
6) In the application of the 50 – 2500 ng/L measuring range, dilute the samples with the sample diluent 10x (cat.nr. HU0040101; 50 µL of the sample + 450 µL of sample diluent); to obtain the effective aflatoxin M_1 concentration in samples, the concentration read from the calibration curve must be multiplied by 10.

6.2. Raw bovine milk

As an alternative option to procedure 6.1, it is possible to analyze raw bovine 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 whole milk directly in the assay, after it is adjusted to room temperature.
Continue from step 4 – 6 of chapter 6.1.

6.3. Powdered bovine milk

1) Weigh 10 g of the powdered milk, add distilled or deionized water, mix and dilute to 100 mL with distilled or deionized water.
2) Shake until the powder is completely dissolved.
3) The dilution factor is 10.

7. WORKING SOLUTIONS PREPARATION

Aflatoxin M_1 standard solutions: ready to use **(shake gently prior to use).**
Enzyme conjugate: 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 dissolve 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 pipet, 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 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

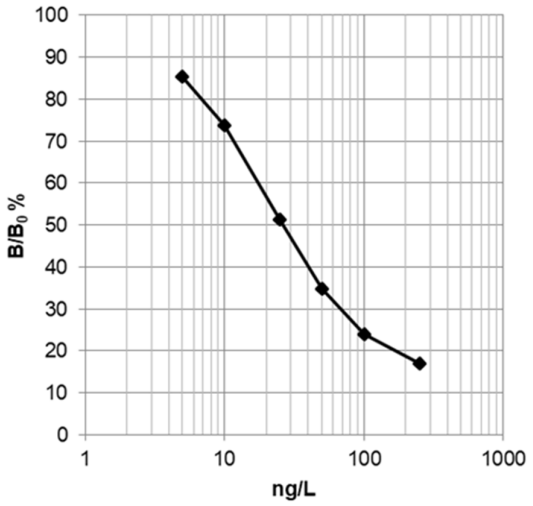
- Calculate the mean absorbance of each standard and sample.
- Divide the mean absorbance value of each standard and sample by the mean absorbance of the standard 0 and multiply by 100; the standard 0 is thus made equal to 100% and all the other absorbance values are expressed as percentages:

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

- 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 by the dilution factor.

Please note: for results calculation it is suggest to use the "point to point" curve. Excel spreadsheet are available in the Document Repository section of the Gold Standard Diagnostics web site (www.goldstandarddiagnostics.com).

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 provided specifications, 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 B_0 absorbance	$\geq$ 0.7 OD_{450nm}
B/B_0 50 %	18 - 40 ng/L
Std duplicates mean C.V.	$\leq$ 6 %

12.2. Assay performance

Raw 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 M_1

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 a confirmatory method (as HPLC, LC-MS, LC-MS/MS).*

13. LIABILITY

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

14. LITERATURE

Bianco E., Bravin F., Tan D., Diana F. Robust detection of aflatoxin M_1 in raw bovine milk without performing any defatting procedure through a sensitive, accurate and precise enzyme immunoassay. Poster presentation at WMF meets ASIA, the 12th Conference of The World Mycotoxin Forum, 2020, January 13-15, Bangkok, Thailandia.

Bianco E., Gon F., Tamburlini F., Diana F.. Verification of the performance of two ELISA test kits for aflatoxin M_1 in milk and dairy products. Poster presentation at WMF meets IUPAC, 2019, October 14-16, Belfast, Northern Ireland.

Sternieri M., Diana F., Persic L. Aflatoxin M_1 analysis of non-skimmed bovine raw milk with I' Screen AFLA M_1 ELISA kit. Poster presentation at 10^{th} World Mycotoxin Forum,2018, march 12-14, Amsterdam, The Netherlands.

Rosar G., Puppini B., Bassani V., Persic L. Monitoring the performances of Tecna's ELISA test kits for mycotoxins through proficiency test participation. Poster presentation at 7th International Symposium on Recent Advances in Food Analysis, 2015, November 3-6, Prague, Czech Republic. ISBN 978-80-7080-934-1.

Diana F., Vascotto F., Rosar G., Persic L. A reliable and well-controlled screening tool: I'screen AFLA M_1 MILK ELISA kit Poster presentation at 6th International Symposium on Recent Advances in Food Analysis, 2013, November 5-8, Prague, Czech Republic. ISBN 978-80-7080-861-0.

Rosi P., Borsari A., Lasi G., Lodi S., Galanti A., Fava A., Girotti S., Ferri E. (2007). Aflatoxin M_1 in milk: Reliability of the immunoenzymatic assay. Int. Dairy J. 17(5): 429-435.

Decastelli L., Lai J., Gramaglia M., Monaco A., Nachtmann C., Oldano F., Riffier M., Sezian A., Bandirola C. (2007). Aflatoxins occurrence in milk and feed in Northern Italy during 2004-2005. Food Control 18(10): 1263-1266.

Rosi P., Borsari A., Lodi S., Galanti A., Fava A. Determinazione del contenuto di aflatossina M_1 nel latte: correlazione fra metodo immunoenzimatico (ELISA) e cromatografico (HPLC-FLD). Sessione poster. I Convegno nazionale micotossine nella filiera agro-alimentare. 2004. 29-30 novembre 2004. Roma. Italia.

Amatiste S., Rosati R., Ubaldi A., Proietti A., Pietrini P., Giangolini G., Brizoli N.R. Gestione dell'emergenza aflatossine nelle regioni Lazio: Metodi di analisi impiegati per latte e alimenti zootecnici. Sessione poster. Convegno Agrofood Bioanalysis I. 2004. 24-26 giugno 2004. Alghero. Italia.

Focardi C., Nocentini M., Palmerini F., Posolini I., Vitullo A. Aflatossina M_1 nel latte: confronto tra metodo di screening ELISA e metodo di conferma in HPLC. Atti del I Convegno Agrofood Bioanalysis I. 2004. 24-26 giugno 2004. Alghero. Italia.