BZERO AFLA M1

Enzyme immunoassay for the detection of Aflatoxin M1 (Cat.nr. HU0040003 / HU0040023)

BZERO **AFLA M1** is a kit prepared for a competitive immunoenzymatic assay for the quantitative analysis of aflatoxin M1.
The kit contains the procedure and the materials sufficient for 96 determinations ***(Cat.nr. HU0040003)*** or 48 determinations ***(Cat.nr. HU0040023)*** 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)
Raw milk (from cow, sheep, goat), UHT milk, yogurt.

Sample preparation
- Milk: direct analysis
- Yogurt: extraction with solvent, centrifugation, evaporation, reconstitution.

Assay time: 30 minutes.

Detection limit
10ng/L

Specificity	
Compound	**Cross-reactivity (%)**
Aflatoxin M_1	100
Aflatoxin M_2	<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 solution 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/standard.

2. PROVIDED REAGENTS

Microtiter plate: 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.
Std. 0: plastic vial containing the 0 ng/l standard of aflatoxin M_1.
ATTENTION: Only the zero standard is provided. B/B_0 values of calibration curve (10-200 ng/l) are reported in the kit certificate of conformity.
Enzyme conjugate: 1 plastic bottle.
Washing-buffer 20X: 1 plastic bottle containing 50 mL.
Developing solution: 1 plastic bottle.
Stop solution: 1 glass bottle. White cap.

Component	Cat.nr. HU0040003 96 det.	Cat.nr. HU0040023 48 det.
Microtiter plate	96 wells (12 strip x 8 wells)	48 wells (6 strip x 8 wells)
Aflatoxin M_1 Std.0	2 vials x 3 mL	3 mL
Enzyme conjugate	14 mL	8 mL
Washing buffer 20x	50 mL	50 mL
Developing solution	14 mL	8 mL
Stop solution	8 mL	6 mL

3. MATERIALS REQUIRED BUT NOT PROVIDED

- Pre-mixing plate.
- Micropipette 50-200 µl, tips.
- Multichannel micropipette 50-250 µl, tips.
- Microplate reader, filter 450 nm.
- **For yogurt analysis:**
  - balance
  - vials
  - methanol
  - chemical hood
  - evaporator
  - shaker
  - vortex
  - Centrifuge or Whatman n.1 filters
  - Additional Components for AFLA M1, yogurt analysis, **Cat.nr. HU0040119**

4. WARNING AND PRECAUTIONS FOR THE USERS

- The test is for *in vitro* diagnostic use only.

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- 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 (at least 1 hour). 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, UHT milk

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

1) Bring the milk to room temperature.
2) Analyse the milk directly in the assay, without skimming.

6.2. Yogurt

1) Weigh 1 g of sample and add 5 mL of methanol.
2) Shake thoroughly for 5 min.
3) Centrifuge for 5 min at 5000xg or, as alternative, filter through Whatman 1 filter.
4) Transfer 1 mL of the upper organic phase or of the filtrate and evaporate at 40 °C under a slow air or nitrogen stream.
5) Dissolve the residue in 0.2 mL of 1x sample buffer (provided in **Cat.nr. HU0040119**) and mix thoroughly for 1 min (vortex).
6) Add 0.2 mL of sample diluent (provided in **Cat.nr. HU0040119**) and mix thoroughly for 30 sec (vortex).
7) The dilution factor is 2.

7. WORKING SOLUTIONS PREPARATION

Std.0: 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 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.

For yogurt analysis:
Sample Buffer: dilute the concentrate solution 1:10 (0.5 mL + 4.5 mL) 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.
Sample diluent: ready to use.

8. ASSAY PROCEDURE

1) Predispose an assay layout, recording standard 0 and samples positions and taking into account that one well is required for each standard and sample.

ATTENTION: If using a monochannel pipette it is adived not to carry out more than 8 determinations (samples and standard 0) in each session; if using a multichannel pipette it is adviced not to carry out more than 24 determinations (samples and standard 0) in each session, if using a multichannel pipette together with a pre-mixing plate, it is possible to carry out up to 48 determinations (samples and standard 0).

2) First incubation
   - Add 100 µl of each sample and standard 0 into the corresponding wells
   - Shake the plate gently with rotatory motion for few seconds
   - Incubate 10 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 three (3) 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
   - Incubate for 5 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.
   - 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 15 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

- Divide the mean absorbance value of each sample by the 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 provided for each standard (10; 25; 75; 200 ng/l) in the kit lot certificate in a semi-logarithmic system of coordinates against the aflatoxin M1 standard concentration and draw the standard curve.
- Take the B/B_0 value for each sample and interpolate it to the corresponding concentration (ng/l) in the calibration curve.

Please note: for results calculation, use "point to point" standard curve Excel spreadsheet are available on Eurofins Tecna website tecna.eurofins-technologies.com and can be downloaded directly from the bottom of the product page.

10.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 11).
If the values are outside the specifications given, then the results of the test are not assured, therefore the aflatoxin M1 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.

11.KIT SPECIFICATIONS

11.1 Assay specification

Description	Specifications
B_0 absorbance	$\geq$ 0.7 OD_{450nm}

11.2 Assay performance

Matrix	Cut off - ng/L	LOQ- ng/L
Raw bovine milk	<10	15
UHT milk	<10	15
Yogurt	<20	40

Matrix	Recovery % ± ds
Bovine milk, spiked reference materials	105 ± 11
Bovine milk, incurred reference materials	103 ± 13

The results were obtained by means of a "point to point" the calibration curve.

12.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.

13.LITERATURE

Rosar G., Diana F. Master curve calibrated assays for cost-effective, reliable and consistent analysis of mycotoxins: a "B ZERO" review. Poster presentation at WMF meets ASIA, the12th Conference of The World Mycotoxin Forum, 2020, January 13-15, Bangkok, Thailandia.

Diana F., Bianco E., Persic L. Direct analysis of aflatoxin M1 in not skimmed milk using B ZERO AFLA M1 ELISA kit. Poster presentation at the RME 2015 10th Conference, 20-22 April 2015, Noordwijkerhout, The Netherlands.