VERSION N2
CAT.NUMBER: B2048/B2096/B20192

Bio-Shield M1 ES

ELISA TEST | In vitro analysis

for the quantitative determination of Aflatoxin M1 in in milk, milk powder, cheese and yogurt

This ELISA kit is manufactured by ProGnosis Biotech S.A. and complies with the specifications on the Standard EN ISO 14675:2003

This ELISA kit has been validated by the Institute for Agricultural, Fisheries and Food Research (ILVO) in Belgium.

ProGnosis Biotech S.A. is ISO 9001:2015 certified by TÜV Hellas (TÜV NORD).

Use only the current version of Product Data Sheet enclosed with the kit.

Bio-Shield M1 ES, B2048/B2096/B20192, is an immunoassay method that determines the Aflatoxin M1 in milk, milk powder, cheese and yogurt. The ELISA kit contains all reagents required for the immunoassay method. The ELISA test is adequate for 48/96/192 definitions (standards are included). A spectrophotometer for microtiter ELISA plate is required.

Sample preparation:

- milk: no preparation,
- milk powder: reconstitution and/or defatting,
- cheese: extraction, evaporation, reconstitution, centrifugation,
- dilution and yogurt: dilution

Test time (incubation time after samples and reagents preparation): 75min

Standard curve range: 0 - 250ppt

Shelf life: 12 months

Storage: 2-8°C

This is an electronic version, please verify always the last one included in the kit.

ILVO
Flanders Research Institute for Agriculture, Fisheries and Food

1. Description

Bio-Shield M1 ES is an ELISA test for the detection of Aflatoxin M1 in milk, milk powder, cheese and yogurt.

2. General Information

Aflatoxins are toxic metabolites of major concern to the dairy industry, generally produced by *Aspergillus fl avus, A. parasiticus* and *A. nomius.* They can have immunosuppressive, mutagenic, teratogenic and carcinogenic effects. Aflatoxins that are ingested by animals in contaminated pellets and forage are biotransformed at the hepatic level into Aflatoxin M1 (AFM1). Aflatoxin is then excreted in this form into the milk used for human consumption and, it is also present in dairy products. AFM1 in milk and milk products is considered to pose certain hygienic risks for human health and as a result there is an established EU limit 0.05 μg/kg (50 ppt).

3. Principle of the Method

The quantitative test is based on the enzyme linked immunosorbent assay principles. The wells of the microtiter strips are coated with AFM1 specific antibodies. AFM1 standards or samples are added into the wells of the microtiter plate. Then, AFM1 of standards or samples (if AFM is present) binds to the coated antibodies. Any unbound AFM1 is removed in a washing step. A detection solution with AFM1-HRP conjugate is added and it binds to the binding sites of coated antibodies that are not already occupied by AFM1 of standards or samples. Any unbound AFM1-HRP conjugate of detection solution is removed in a washing step. A chromogen substrate is added to the wells resulting in the progressive development of a blue colored complex with the detection antibody. The color development is then stopped by the addition of acid turning the resultant final product yellow. The measurement is made photometrically at 450 nm and the intensity of the produced colored complex is indirectly proportional to the concentration of AFM1 present in the samples and standards.

4. Reagents Provided

Bio-Shield M1 ES ELISA kit contains sufficient reagents and materials for 48/96/192 measurements (including standard tests).

Reagents (Store at 2-8ºC)	Quantity for 48 wells	Quantity for 96 wells	Quantity for 192 wells	State	Vial cap color
Single-Break Strip Plate	48 wells	96 wells	2 Single-Break Strip Plates (each 96 wells)	Ready to use (precoated)	-
Sealing film	2 sheets	2 sheets	4 sheets	Ready to use	-
Standards 1-7 (0, 5, 10, 25, 50,100 and 250ppt of AFM1)	7 glass vials (each1.5ml)	7 glass vials (each1.5ml)	7 glass vials (each 3.0ml)	Ready to use	Black
M1 ES Detection Solution	1 plastic vial (6ml)	1 plastic vial (12ml)	2 plastic vials (each 12ml)	Ready to use	Green
Wash Buffer	1 plastic vial (50ml)	1 plastic vial (50ml)	1 plastic vial (50ml)	20X Concentrate (dilute in distilled	White
TMB Substrate	1 plastic vial (6ml)	1 plastic vial (12ml)	2 plastic vials (each 12ml)	Ready to use	Brown
Stop Solution	1 plastic vial (6ml)	1 plastic vial (12ml)	2 plastic vials (each 12ml)	Ready to use	White
AFM1-free milk	1 plastic vial (30ml)	1 plastic vial (30ml)	1 plastic vial (50ml)	Ready to use	White
Yogurt Buffer	1 plastic vial (15ml)	1 plastic vial (15ml)	2 plastic vials (each 15ml)	Ready to use	Red

5. Materials required but not provided

- Centrifuge, Magnetic stirrer, Vortex mixer and Microtiter plate reader fitted with 450 nm filter.
- 100 and 1000μl adjustable single channel micropipettes with disposable tips (a repetitive pipette of 100μl is preferable for the steps of Detection Solution, TMB and Stop Solution).
- 50 - 300μl multi-channel micropipette with disposable tips and reservoirs.
- Distilled water, methanol, dichloromethane, n-hexane and pepsin.

6. Storage Instructions

Store kit reagents between 2 and 8°C (35 - 46°F). Do not freeze any components provided. Reseal immediately the unused strips of the microtiter plate in the bag together **with the desiccant bag** provided and store at 2 - 8°C. After use remaining reagents should be returned to cold storage (2 - 8°C). Expiry of the kit and reagents is stated on the labels respectively and no quality guarantee is accepted after the expiration date. The expiry of the kit components can only be guaranteed if the components are stored properly as well as if the reagent is not contaminated by the first handling, in case of repeated use of one component. Because of the colorless TMB Substrate and standards 1-7 light sensitivity, avoid the exposure to direct light. Do not interchange individual reagents between kits of different lot numbers.

7. Safety and Precautions for use

- Avoid any skin contact with standards (AFM1), Stop Solution (15% H_3PO_4) and TMB (toxic). **Use gloves.** In case of contact, wash thoroughly with water.
- All reagents should be warmed in room temperature before use and covered when not in use. **Use a clean disposable plastic pipette tip for each reagent, in order to avoid cross contamination. When pipetting reagents, maintain a consistent order of addition from well-to-well. This will ensure equal incubation times for all wells.**
- Use a clean plastic container to prepare the wash buffer and all residual washing liquid must be drained from the wells by efficient aspiration or by decantation followed by tapping the plate forcefully on absorbent paper. Never insert absorbent paper into the well. Read the absorbance within 60 minutes after completion of the assay.

8. Indication of corruption of kit reagents

- The bluish coloration of the chromogen substrate before the ELISA test.
- A value of less than 0.7 absorbance units (ABS 450nm) for the Standard 1 (St1).

9. Sample and reagents preparation

9.1 Reagents preparation

Dilute the 20X solution concentrate 20 fold with distilled water to give a **1X** working solution.

Preparation of Wash Buffer 1X: In case of the occurrence of crystals in the Wash Buffer, the warming by gentle dismantling (using hands) of the crystals is needed. Pour entire content of the solution concentrate (50ml) into a clean 1000ml graduated cylinder, rinse the vial with distilled or deionised water and pour the content again into the cylinder and fill to a final volume of 1000ml with distilled or deionised water. Mix gently to avoid foaming, transferring the final solution from cylinder to a clean bottle and back two times. The clean bottle with **1X Wash Buffer** working solution can be left out of the refrigerator during the method procedure and subsequent be stored 2 - 8°C for one month.

9.2 Samples preparation

9.2.1 Milk

Use 100μl of each milk sample directly in the immunoassay. Centrifugation (3000xg for 10 min) is not necessary because there is no significant difference in the final result. **The dilution factor is 1.**

9.2.2 Milk Powder

Reconstitute the milk powder according to manufacturer's instructions. If there are no instructions available mix 1g of milk powder with deionized or distilled water until 10ml. Mix well and afterwards there follows the skimming according to the sample preparation of milk (see 9.1). Use 100μl of each sample directly in the immunoassay.

When the milk powder refers to reconstituted milk, **the dilution factor is 1.**

When the milk powder refers to g-weight, **the dilution factor is 10.**

9.2.3 Cheese / Butter

9.2.3.1 Dichloromethane (DCM) Method for Cheese and Butter

Extraction: Weight 2g of a representative sample (finely ground and not from the surface) into a screw cap centrifugal glass vial and add 8ml DCM. While shaking/stirring, incubate at room temperature (19-24°C) for 30min. Centrifuge the suspension for 10min at 3000xg at room temperature. Transfer 4ml of the extract and evaporate at 60°C under a weak N_2-stream. Redissolve the oily residue in 0.5ml methanol 100%, 0.5ml distilled water and add 2ml hexane for degreasing. Mix thoroughly and centrifuge again for 10min at 3000xg. Remove the upper hexane-layer and pour off the lower methanolic-aqueous phase using a pasteur pipette. Dilute a part of it 1:10 (1+9) with AFM1-free milk (e.g. dilute 50μl with 450μl AFM1-free milk). Use 100μl of each sample directly in the immunoassay. **The dilution factor is 10.**

9.2.3.2 Pepsin Method for Cheese

Weight 2.5g of a representative cheese sample (finely ground and not from the surface) into a 50ml screw cap centrifuge tube and add 25ml of 0.2% pepsin in 0.1N HCl. Incubate for 16h in an incubator at 42ºC with shaking or stirring. Centrifuge for 15min at 4000xg at room temperature. Pass the supernatant through a Whatman #1 filter paper (or equivalent) to remove the fat residue and collect the filtrate. In 10ml of filtrate add 0.2ml of 5N NaOH and make sure that the pH is 7-7.5. Dilute 1:1 (1+1) with AFM1-free milk (e.g. dilute 0.5ml with 0.5ml AFM1-free milk). Use 100μl of each sample directly in the immunoassay. **The dilution factor is 20.**

9.2.4 Yogurt Samples

9.2.4.1 Yogurt

In a 15ml tube add 1g of yogurt sample, 0.5mL of deionized water and 1.5ml of Yogurt Buffer. Mix well by vortex (15 sec). Use 100μl of each sample mixture directly in the immunoassay.
The dilution factor is 3.

9.2.4.2 Yogurt Drink

In a 15ml tube add 1mL (or 1g) of yogurt drink sample and 1mL of Yogurt Buffer. Mix well by vortex (15 sec). Use 100μl of each sample mixture directly in the immunoassay. **The dilution factor is 2.**

10. Method Procedure

10.1 Assay Design: Determine the number of microwell strips required to test the desired number of samples plus appropriate number of wells needed for standards. Considering that each sample and standard can be tested in single or in duplicate, create a layout. **NOTE:** If the number of wells is more than 32 (four strips), a repetitive pipette or multichannel pipette is necessary, in case of Detection Solution, TMB Substrate and Stop Solution.

CAUTION: Use the standards positions in duplicate as the **Example plate** layout below **NECESSARY** and note positions of samples that can be set to all remaining empty wells of layout in duplicate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	St1	St1										
B	St2	St2										
C	St3	St3										
D	St4	St4										
E	St5	St5										
F	St6	St6										
G	St7	St7										
H												

10.2 Bring all reagents to room temperature (19 - 24°C) before use. Remove the **standards** (Standard 1-7) and the **appropriate number of wells** into the holder of microwells for the standards and the samples to be worked in duplicate. Place the wells into the holder of microwells and immediately reseal the unused strips of the microtiter plate in the bag together with the desiccant bag provided.

10.3 The samples should be stored in a cool place. Add **100μl** per well of each standard **(Standard 1 - 7)** or prepared sample (see Chapter 9) in duplicate. If the number of wells is more than 64 (eight strips), it is advised to use dilution microwells and a multichannel pipette. Cover the microwells with the sealing film, shake the microplate manually for 30 seconds and incubate at room temperature for **45min.**

10.4 Remove the sealing film and wash the plate as follows: Aspirate the liquid from each well into the sink and tap the holder of microwells upside down strongly (four times in a row) on an absorbent paper to insure the complete removal of liquid from the wells. Dispense **300μl** of **Wash Buffer 1X** (see 10.4) into each well with wash bottle or multichannel micropipette using the proper reagent reservoir and shaking the plate manually for a few seconds. Repeat this process for another three times **(total 4 times). CAUTION:** It is important to not allow microwells to dry between working steps.

10.5 Aspirate the liquid as described above and add **100μl** of **Detection Solution** to each well. If the number of wells is more than 32 (four strips), a repetitive pipette or multichannel pipette is necessary (pour 1 ml of Detection Solution in a reservoir per 8 wells). Cover the microwells with the sealing film, shake the plate manually for a 30 seconds and incubate at room temperature for **15min.**

10.6 Remove the sealing film and wash the plate as the wash **step 10.4.**

10.7 Aspirate the liquid as described above and add **100µl** per well of **TMB Substrate** (pour 1ml per 8 wells in a reservoir). Cover the microwells with the sealing film, shaking the plate manually for a few seconds and incubate in the dark at room temperature for **15min.**

10.8 Remove the sealing film and add **100µl** per well of the **Stop Solution** to each well (pour 1ml per 8 wells in a reservoir). Mix gently by shaking again the plate manually.

10.9 **Measure the absorbance at 450nm.** Read the absorbance value of each well (within 60 minutes after the step 10.8) on a spectrophotometer using 450nm as the primary wavelength and optionally 620 nm as the reference wave length (610 nm to 650 nm is acceptable).

11. Data Analysis

- **Automatically**

An assigned software, the **Prognosis-Data-Reader,** is available for free (contact: info@prognosis-biotech.com) download in order to evaluate the Bio-Shield M1 ES ELISA kit. The evaluation is carried out by a simple transfer of data values after the measurement.

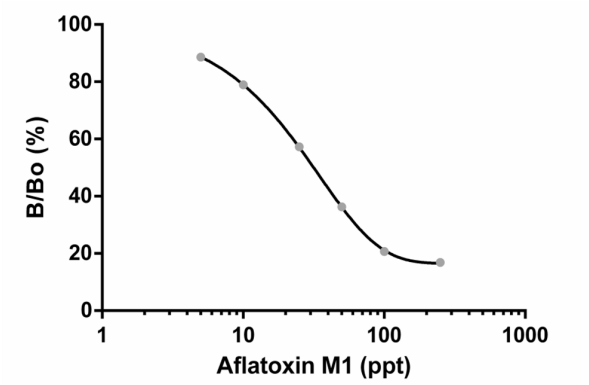
- **Manually**

Calculate the average absorbance values for each set of duplicate standards and samples. Ideally duplicates should be within 10% of the mean. Use the following calculation:

$$\frac{\text{Standard or sample absorbance}}{\text{Standard 1 absorbance}} \times 100 = \%\ \text{Binding}$$

The standard 1 is equal to 100 % and the absorbance values are quoted in percentages. The concentration of Aflatoxin M1 (ppt) in each sample is determined by extrapolating OD values against concentrations of Aflatoxin M1 in standard solutions using a two phase exponential decay standard curve with logarithmic X axis.

12. Example of Standard Curve (0 - 250ppt)



13. Immunoassay Specification

13.1 General Specification

- Mean of Bo (St1) absorbance (ABS 450nm) > 0.7
- IC50 = 15- 45 ppt
- Each standards duplicates mean CV ≤ 6%
- Precision (range 0 - 250ppt): Intrassay CV < 5% and Interassay CV <10%
- Specificity: Cross-reaction of the anti-Aflatoxin M1 antibody with AFM1 and AFM2 is 100 and <0.1% respectively. The anti-Aflatoxin M1 antibody has no cross-reactions with other mycotoxins (Ochratoxin A, Zearalenone, Deoxynivalenol and Fumonisin B1) and other unrelated compounds, such as antibiotics (Benzylpenicillin, Cefalonium, Oxytetracycline, Erythromycin, Neomycin, Enrofloxacin, Sulfadiazine, Trimethoprim and Dapsone).

13.2 LOD - LOQ - Accuracy

		Raw and homogenized milk	Whole milk powder	Cheese / Butter	Cheese	Yogurt Samples	
				DCM Method	Pepsin Method	Yogurt	Yogurt Drink
LOD		2ppt	2ppt*	2ppt*			
LOQ		5ppt	5ppt*	5ppt*			
Accuracy (of result)	Recovery (concentrations between 10 and 75ppt of AFM1)	100% ± 20%	100% ± 20%	100% ± 20%			

* milk reconstituted or diluted

14. Performance Evaluation

14.1 Reference Materials

Several reference materials are being used for the evaluation of each product of ProGnosis Biotech S.A. in the context of Quality Control performed by Quality Control Department. Please request a validation report, including the results, at info@prognosis-biotech.com.

14.2 Proficiency Tests

All products participate frequently in Proficiency Tests. For more information, visit the individual product page in our website: **www.prognosis-biotech.com**

15. Method Summary

Total procedure time (after samples and reagents preparation): 75 min.

Add 100 µl of each standard and sample in microplate

⇩

Incubate 45min at room temperature

⇩

Wash four times

⇩

Add 100 µl of Detection Solution

⇩

Incubate 15min at room temperature

⇩

Wash four times

⇩

Add 100 µl of TMB Substrate

⇩

Let the color develop for 15min in the dark at room temperature

⇩

Add 100 µl Stop Solution

⇩

Read Absorbance at 450 nm within 60 min

All immune assays supplied by ProGnosis Biotech S.A., are warranted to meet or exceed our published specification when used under normal conditions in your laboratory. If the product fails during the stated period, a replacement product will be issued.

ProGnosis Biotech S.A. makes no warranty of any kind, either expressed or implied, except that the materials from which its products are made are of standard quality. There is no warranty of merchantability of this product, or of the fitness of the product for any purpose. ProGnosis Biotech S.A. shall not be liable for any damages, including special or consequential damage, or expense arising directly or indirectly from the use of this product. This method is considered to be a screening method, before a legal action, samples detected as positives must be confirmed with a confirmation method. This product is meant to be used only For Research or Manufacturing use and by qualified technicians.

VERSION N2

CAT.NUMBER: B2049/B2096B20192

STORAGE: 2-8°C

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