BIO-SHIELD M1 BF

B3048 / B3096

ELISA test for the quantitative determination of Aflatoxin M1 in milk, milk powder, cheese and yoghurt

In vitro analysis
Storage at 2-8^{o}C

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

Prognosis Biotech is ISO 9001 certified by TÜV Hellas (TÜV NORD).

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

Bio-Shield M1 BF (Baby Food), B3048/B3096, is an immunoassay method that determines the Aflatoxin M1 in milk, milk powder and cheese. The ELISA kit contains all reagents required for the immunoassay method. The ELISA test is adequate for 96 definitions (standards are included). A spectrophotometer for microtiter ELISA plate is required.

- Sample preparation: milk: defatting, milk powder: reconstitution and/or defatting, cheese: extraction, evaporation, reconstitution, centrifugation, dilution and yogurt: extraction, evaporation, reconstitution
- Test time (incubation time after samples and reagents preparation): 75 min
- Standard curve range: 0 - 80ppt
- Shelf life: 12 months
- Storage: 2-8^{o}C

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1. Description

Bio-Shield M1 BF (Baby Food) is an ELISA test for the detection of Aflatoxin M1 in milk, milk powder, cheese and yoghurt.

2. General Information

Aflatoxins are toxic metabolites of major concern to the dairy industry, generally produced by Aspergillus flavus, 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 the EU limit is 0.05 µg/kg (50ppt) and the limit for baby food is 0.025 µg/kg (25ppt).

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 AFM1 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 BF ELISA kit contains sufficient reagents and materials for 96 measurements (including standard tests).

Reagents (Store at 2-8ºC)	Quantity for 48 wells	Quantity for 96 wells	State	Vial cap color
Single-Break Strip Plate	48 wells	96 wells	Ready to use (precoated)	-
Standards 1-6 (0, 5, 10, 20, 40, 80ppt of AFM1)	6 glass vials (each 1.5ml)	6 glass vials (each 1.5ml)	Ready to use	Black
M1 BF Detection Solution	1 plastic vial (6ml)	1 plastic vial (12ml)	Ready to use	Green
Wash Buffer	1 plastic vial (50ml)	1 plastic vial (50ml)	20X Concentrate (dilute in distilled water)	White
TMB Substrate	1 plastic vial (6ml)	1 plastic vial (12ml)	Ready to use	Brown
Stop Solution	1 plastic vial (6ml)	1 plastic vial (12ml)	Ready to use	White
AFM1-free milk	1 plastic vial (30ml)	1 plastic vial (30ml)	Ready to use	White

5. Materials required but not provided

- Centrifuge, Vortex mixer and Microtiter plate reader fitted with 450 nm filter

- 100 and 1000 µl adjustable single channel micropipettes with disposable tips (a repetitive pipette of 100µl is preferable for the steps of Detection Solution, TMB and Stop Solution)
- 50-300 µl multi-channel micropipette with disposable tips and reservoirs
- Distilled water, methanol for cheese and yoghurt and dichloromethane and n-hexane for cheese

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 colourless 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 (A450 nm) for the Standard 1 (St1).

9. Sample preparation

9.1 Milk

Each fresh cold (2-8°C) milk of 2-15ml to be tested are pipetted into test centrifuge tubes respectively and there follows a centrifugation at 3000xg for 10 min at 2-8°C. Remove the upper fat layer from skimmed milk using a spatula. Use 100µl of each skimmed milk directly in the immunoassay

9.2 Milk Powder

Reconstitute the milk powder according to manufactures instructions. If there are no instructions available mix 1g of milk powder with 10ml deionised or distilled water. 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.

9.3 Cheese

Weight 2g of a representative cheese sample (finely ground and not from the surface) into a screw cap centrifugal glass vial and add 8ml dichloromethane. Extract by stirring/shaking the vial and incubate at 50 °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 N2-stream. Redissolve the oily residue in 500µl methanol 100%, 500µl 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.

9.4 Yoghurt

Weight 1g of sample and add 5 ml of methanol. Shake thoroughly for 5min and centrifuge for 5min at 3000xg. Transfer 250µl of the upper organic phase and evaporate at 40 °C under a slow air or nitrogen stream. Dissolve the residue in 250µl of AFM1-free milk and mix for 1min by vortex. Let it stay for 5min. Use 100µl of each sample directly in the immunoassay. The dilution factor is 5.

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 should be tested 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.

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												
H												

Example plate layout (example for a 6 point standard curve)

10.2 Bring all reagents to room temperature (19-24°C) before use. Remove the **standards** (Standard 1-6) 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 immidiately 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 **100 µl** per well of each standard **(Standard 1 - 6)** or prepared sample (see Chapter 9) in duplicate. Cover the microwells with the sealing film, shake the microplate manually for 30 seconds and incubate at room temperature for **45min.** During this step, prepare the Wash Buffer 1X working solution (see step 10.4).

10.4 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 1000 ml 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.

10.5 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.

10.6 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 **15 min.**

10.7 Remove the sealing film and wash the plate as the wash **step 10.5.**

10.8 Aspirate the liquid as described above and add **100 µl** 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 **15 min.**

10.9 Remove the sealing film and add **100 µl** 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.

10.10 **Measure the absorbance at 450 nm.** Read the absorbance value of each well (within 60 minutes after the step 10.9) on a spectrophotometer using 450 nm 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 by free download from www.prognosis-biotech.com in order to evaluate the Bio-Shield M1 BF ELISA kit. The evaluation is carried out by a simple transfer of data values after the measurement.

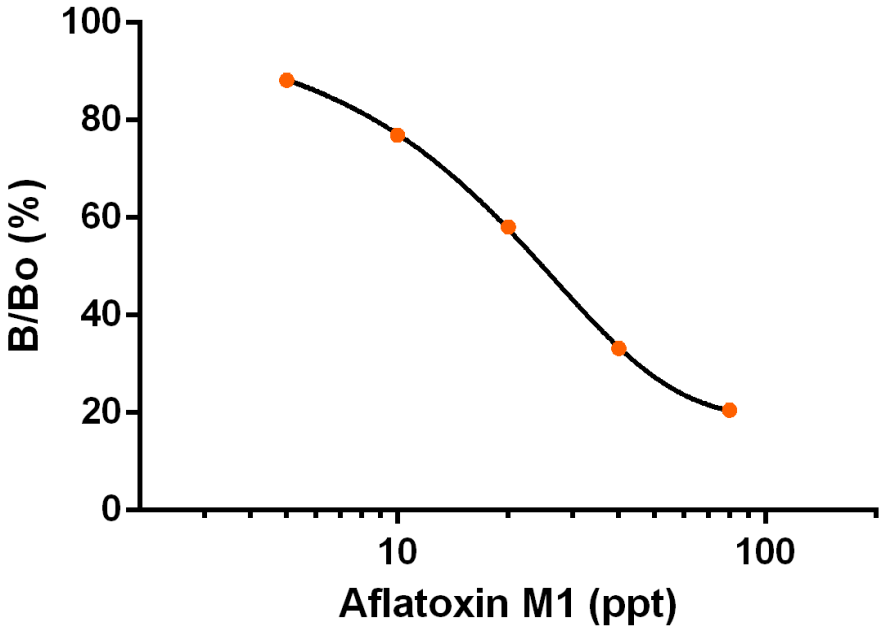
- Manually

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 (0-80ppt)



13. Immunoassay Specification

13.1 General Specification

- IC_{50} = 12.5-40 ppt
- Each standards duplicates mean CV ≤ 6%
- Precision (range 0-80ppt): Intrassay CV < 5% and Interassay CV <10%
- Specificity: Cross-reaction of the anti-Aflatoxin M1 antibody with Aflatoxin M2 is <0.1%.

13.2 LOD - LOQ - Accuracy

		Milk	Milk powder	Cheese	Yoghurt
LOD		1.6ppt	1.6ppt	16ppt	8ppt
LOQ		5ppt	5ppt	50ppt	25ppt
Accuracy (of result)	Recovery	98%	105%	99%	99%
	Satisfactory Range	76-120%	83-127%	77-121%	77-121%

14. Performance Evaluation

14.1 Reference Materials

Reference Material	Lot Number	Certified value (µg/kg)	Uncertainty (µg/kg)	Result (µg/kg)
Test Veritas MI1460-1/CM	Progetto Trieste 2014, II round mycotoxin 2014	0.407	0.178	0.385

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

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