BIO-SHIELD B1

B1548 / B1596

ELISA test for the quantitative determination of Aflatoxin B1 in grains, cereals, spices and animal feeds

In vitro analysis

Storage at 2-8 ºC

www.prognosis-biotech.com

This ELISA kit is manufactured by: ProGnosis Biotech Ltd.

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 B1, B1548/B1596, is an immunoassay method that determines the Aflatoxin B1 in grains, spices, cereals and other commodities including animal feeds. The ELISA kit contains all reagents required for the immunoassay method. The ELISA test is adequate for 96/48 definitions (standards are included). A spectrophotometer for microtiter ELISA plate is required.

- Sapmle preparation: extraction
- Test time (incubation time after samples and reagents preparation): 20 min
- Standard curve range: 0 – 30ppb
- Shelf life: 12 months
- Storage: 2-8^{o}C

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1. Description

Bio-Shield B1 is an ELISA test for the detection of Aflatoxin B1 (AFB1) in grains, spices, cereals and animal feeds.

2. General Information

Aflatoxins are toxic metabolites of major concern to the food industry, generally produced by Aspergillus flavus, A. parasiticus and A. nomius. They can have immunosuppressive, mutagenic, teratogenic and carcinogenic effects. Aflatoxins can be present in grains, spices, cereals and other commodities associated with human food or animal feed. Crops may be contaminated with AFB1. AFB1 is the most toxic and frequently detected form. The other types present a significant danger if the concentration is at a high level. Animals are exposed to aflatoxins by consumption of feeds that have fungal strains producing aflatoxins during growth, harvest or storage. Symptoms of toxicity in animals range from death to chronic diseases, reproductive interference, immune suppression, decreased milk and egg production. Most controlling government agencies worldwide have regulations regarding the amount of aflatoxins allowable in human and animal foodstuffs. Accurate and rapid determination of aflatoxin presence in commodities is of paramount importance.

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 AFB1 specific antibodies. Toxins are extracted from a ground sample with 70% methanol. AFB1 standards or samples and AFB1-HRP conjugate (detection solution) are added into the coated wells. AFB1-HRP conjugate binds to the binding sites of coated antibodies that are not already occupied by AFB1 of standards or samples. Any unbound AFB1-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 AFB1 present in the samples and standards.

4. Reagents Provided

Bio-Shield B1 ELISA kit contains sufficient reagents and materials for 48 or 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)	-
Dilution Microwells	48 wells	96 wells	Ready to use (red color)	-
Matrix Diluent	1 plastic vial (12ml)	2 plastic vials (12ml)	Ready to use	Red
Standards 1-6 (0, 0.2, 0.5, 1, 2 and 6 ppb of AFB1 in organic solution) (correspond to 0, 1, 2.5, 5, 10 and 30ppb)	6 glass vials (each 1.5ml)	6 glass vials (each 1.5ml)	Ready to use	Black
B1 Detection Solution	1 plastic vial (6ml)	1 plastic vial (12ml)	Ready to use	Green
Wash Buffer	1 plastic vial (50ml)	1 plastic vial (50 ml)	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

5. Materials required but not provided

- A grinder sufficient to render sample to particle size of fine instant coffee
- Balance with 0-50g measuring capability and Graduated cylinder - 100mL
- Methanol (70mL reagent grade per sample) and Distilled or deionized water
- Filter Paper Whatman #1 or equivalen, Filter Funnel and Miscellaneous laboratory plastic or glass tubes 50-125ml
- Vortex mixer and Microtiter plate reader fitted with 450 nm filter
- 100, 200 and 1000 µl adjustable single channel micropipettes with disposable tips (a repetitive pipette of 100µl is acceptable for the steps of TMB and Stop Solution)
- 50-300 µl multi-channel micropipette with disposable tips and reservoirs

6. Storage Instructions

Store kit reagents between 2 and 8°C. 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 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 AFB1 standards, 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 30 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

- Prepare the Extraction Solution (70% Methanol) by adding 30mL of distilled or deionized water to 70mL of methanol (reagent grade) for each sample to be tested.
- The sample must be collected according to established sampling techniques. Grind

a representative sample to the particle size of fine instant coffee (50% passes through a 20 mesh screen).

• Weigh out a 20g ground portion of the sample and add 100mL of the Extraction Solvent (70% methanol) and mix in a blender for a minimum of 2 minutes. **The ratio of sample to extraction solvent is 1:5 (w/v).**

• Allow the particulate matter to settle, then filter 5-10mL of the extract through a Whatman #1 filter paper (or equivalent) and collect the filtrate. Use 50µl of each filtrte directly in the immunoassay.

NOTE 1: The extracted sample should have pH value of 6.2-7.5. If the pH is less than 6.2 as for example happens on the the silage samples, the pH should be neutralized using NaOH.

NOTE2: In case the user make an additional dilution 1:1 of filtrate with 70% methanol the range of quantification becames 0-60ppb. So, use also 50µl of each diluted filtrate directly in the immunoassay and multiply the final AFB1 ppb result x 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 should be tested in duplicate, create a layout. **NOTE:** Do not use more than 48 wells (six strips) in a single experiment.

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 place two Dilution Microwells (red) in a microwell holder for each Standard and Sample to be tested in duplicate. Place an equal number of Antibody Coated Microtiter Wells in another microwell holder. Immidiately reseal the unused strips of the microtiter plate in the bag together with the desiccant bag provided. The samples should be stored in a cool place.

10.3 Add **200µl** of **Matrix Diluent** to each Dilution Well.

10.4 Using new pipette tip for each, add **50µl** of each Standard **(Standard 1 - 6)** and prepared sample in duplicate (see Chapter 9) to appropriate Dilution Well containing the Matrix Diluent. Mix by priming pipetting at least 5 times.

10.5 Using a multichannel pipette, transfer **100µl** of contents from each Dilution Microwell to a corresponding Antibody Coated Microtiter Well. Cover the microwells with the sealing film and incubate at room temperature for **10 minutes**.

10.6 **DO NOT WASH THE WELLS BEFORE ADDING THE DETECTION SOLUTION.** Remove the sealing film. Using a multichannel pipette, add **100µl** of **Detection Solution** to each well and mix by priming pipetting once (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 **5 min. NOTE:** During this step, prepare the Wash Buffer 1X working solution (see step 10.7).

10.7 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.

10.8 Remove the sealing film and wash the plate as follows: Aspirate the liquid from each well (200µl/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 step 10.7) 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.

10.9 Aspirate the liquid from each well and tap the holder of microwells upside down strongly on the absorbent paper as described above and add **100 µl** per well of **TMB Substrate.** Cover the microwells with the sealing film, shaking the plate manually for a few seconds and incubate in the dark at room temperature for **5 min.**

10.10 Remove the sealing film and add **100 µl** per well of the **Stop Solution** to each well. Mix gently by shaking again the plate manually.

10.11 **Measure the absorbance at 450 nm.** Read the absorbance value of each well (immediately after the step 10.10) 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 B1 ELISA kit. The evaluation is carried out by a simple transfer of data values after the measurement.

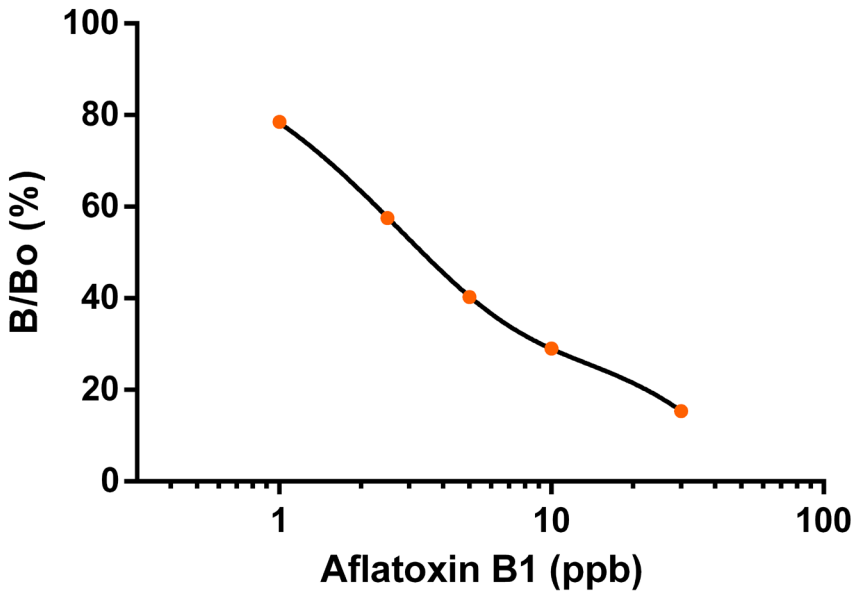
- Manually

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 B1 (ppb) in each sample is determined by extrapolating OD values against concentrations of Aflatoxin B1 in standard solutions using a two phase exponential decay standard curve with logarithmic X axis.

12. Example of Standard Curve (0-30ppb)



13. Immunoassay Specification

13.1 General Specification

- IC_{50} = 1.3-5.3 ppb
- Each standards duplicates mean CV ≤ 6%
- Coefficient of Variation (CV) of result at 2ppb = 12.8% (n=16)
- Coefficient of Variation (CV) of result at 5ppb = 10.0% (n=16)

13.2 LOD - LOQ - Recovery

- The LOD of the method is 0.3ppb
- The LOQ of the method is 0.7ppb
- The recovery of spiked extractions-matrices (Barley, Coconut, Corn, Silage, Cottonseed, DDGs, Oats, Sorgum, Soy, Wheat, Rye, Barley, Corn flakes, Oat bran, Bran sticks, Rice, Soy, Oat flakes, Oat, Lolium, Peanuts, Paprika, Hazelnut, Chickpeas, Pepper, Pet Food, Sunflower, Dates, Dry figs, Almond Butter, Peanut Butter and green coffee) was 101.2% (CV = 10.5%).

13.3 Specificity

The cross-reaction of the anti-Aflatoxin B1 antibody with Aflatoxin B1, B2, G1 and G2 is 100, 7, 18 and 1% respectively.

14. Performance Evaluation

Reference Material	Lot Number	Certified value (µg/kg)	Uncertainty (µg/kg)	Result (µg/kg)
FAPAS Maize Reference T04246QC	Nº 105	4.91	2.17	5.09
ERM®-BE375 feedingstuff material	Nº 0083	2.6	0.4	2.78

15. Method Summary

Total procedure time (after samples preparation): 20min.

Mix 200µl of the Matrix Diluent with 50µl of the samples and standards in the Dilution Microwells

↓

Transfer 100µl from each well of the Dilution Microwells into the Antibody Coated Microwells

↓

Incubate 10 min at room temperature

↓

DO NOT WASH / Add 100 µl of Detection Solution

↓

Incubate 5 min at room temperature

↓

Wash four times

↓

Add 100 µl of TMB Substrate

↓

Let the color develop for 5 min in the dark at room temperature

↓

Add 100 µl Stop Solution

↓

Read Absorbance at 450 nm

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PROGNOSIS BIOTECH

ProGnosis Biotech Ltd

R&D, In vitro diagnostics, Biotechnology Marketplace

Iroon Polytechniou 71

Larissa, Greece, 41222

tel: 2410 623922 / 2310 952738

fax: 700 700 6262

WEB: WWW.PROGNOSIS-BIOTECH.COM

E-MAIL: INFO@PROGNOSIS-BIOTECH.COM