| VERSION 14

CAT.NUMBER: D4948

STORAGE: 2-8°C

1 STANDARD AFLATOXIN B1

ELISA TEST | In vitro analysis

for the quantitative determination of Aflatoxin B1 in grains, nuts, cereals, and animal feed

PROGNOSIS BIOTECH

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

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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.

1 Standard B1, D4948, is an immunoassay method that determines the Aflatoxin B1 in grains, nuts, cereals and other commodities including animal feed. The ELISA kit contains all reagents required for the immunoassay method. The ELISA test is adequate for 48 definitions (Zero Standard (St1) is included). A spectrophotometer for microtiter ELISA plate is required.

Matrices:

- **Cereals:** Barley, Biscuits, Bran Sticks, Brown rice, Corn, Corn flakes, Corn flour, Corn Germ, Corn Gluten, Corn Grits, Corn Meal, Corn/Soy blend, Dried Sunflower, Oat bran, Oat flakes, Oats, Popcorn, Raw Rye, Rice, Rye Flour, Sesame, Sorghum, Soy beans, Wheat, Wheat bran, Wheat flour
- **Dried fruit:** Dried Figs
- **Nuts:** Almond, Peanut butter, Raw peanuts, Salted Peanuts
- **Other:** Milk, Oil (Coconut oil, Corn oil, Olive oil, Olive pomace oil, Peanut oil, Sesame oil, Soybean oil, Sunflower oil)

- Sample preparation: extraction
- Test time (incubation time after samples and reagents preparation): 5min
- Standard curve range: 0 - 50ppb
- Shelf life: 12 months
- Storage: 2-8^{o}C

1. Description

1 Standard B1 is an ELISA test for the detection of Aflatoxin B1 (AFB1) in grains, nuts, cereals and animal feed.

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 feed 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

1 Standard B1 ELISA kit contains sufficient reagents and materials for 48 measurements (including Zero Standard test). Zero Standard (St1) is the only standard provided and the B/Bo values of St2-St5 (2 - 50 ppb) are reported in the Quality Assurance Certificate of each lot.

Reagents (Store at 2-8ºC)	Quantity for 48 wells	State	Vial cap color
Single-Break Strip Plate	48 wells	Ready to use (precoated)	-
Dilution Microwells	48 wells	Ready to use (green color)	-
Standard 1 (0ppb)	1 glass vial (3ml)	Ready to use	Black
1 Standard B1 Detection Solution	1 plastic vial (6ml)	Ready to use	Green
Wash Buffer	1 plastic vial (50ml)	20X Concentrate (dilute in distilled water)	White
TMB Substrate	1 plastic vial (6ml)	Ready to use	Brown
Stop Solution	1 plastic vial (6ml)	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 equivalent, Filter Funnel and Miscellaneous laboratory plastic or glass tubes 50 - 125ml
- Vortex mixer and Microtiter plate reader fi ted 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 (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 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 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 (450nm) for the Standard 1 (St1).

9. Sample and reagents preparation

9.1 Reagents 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.
- 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 Ground Samples

1. 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).
2. 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).**
3. 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 100µl of each filter 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 silage samples, the pH should be neutralized using NaOH.

NOTE 2: In case the user make an additional dilution 1:1 of filtrate with 70% methanol the range of quantification becomes 0 - 100ppb. So, use also 100µl of each diluted filtrate directly in the immunoassay and multiply the final AFB1 ppb result x 2.

9.3 Milk Samples

1. Centrifuge the sample at 3,000 g at 4°C for 10 min and remove the upper-fat layer.
2. Dilute the defatted milk sample 5 times with 70% methanol (**1ml of milk + 4ml of 70% methanol**) and vortex.
3. Use 100µl directly in the immunoassay.
4. The final AFB1 ppb result needs no additional calculation.

9.4 Oil Samples

1. Prepare extraction solvent (70% methanol) by adding 30ml of distilled or deionized water to 70ml of methanol for each sample to be tested.
2. Transfer 100ml of extraction solvent to a container and add 20ml of the sample. The ratio of sample to extraction solvent is 1:5 (v/v).
3. Mix in a blender for a minimum of 10 minutes.
4. Centrifuge a portion of the mixture at 3,000 g for 10 min and collect the upper layer (methanol).
5. Use 100µl directly in the immunoassay.
6. The final AFB1 ppb result needs no additional calculation.

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 zero standard. Considering that each sample and standard can be tested in single or in duplicate, create a layout.

NOTE: It is preferred to use no more than 32 wells (four strips) in each assay.

	1	2	3	4	5	6	7	8	9	10	11	12
A	St1											
B												
C												
D												
E												
F												
G												
H												

Example plate layout

10.2 Bring all reagents to room temperature (19 - 24°C) before use. Remove the **standard** (Zero Standard) and place **the appropriate number** of Dilution Microwells (green) in a microwell holder for each Standard and Sample to be tested. Place an equal number of Antibody Coated Microtiter Wells in another microwell holder. Immediately 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 **100µl** of **1 Standard B1 Detection Solution** to each Dilution Well.

10.4 Using new pipette tip for each, add **100µl** of **Standard 1** and prepared sample (see Chapter 9) to appropriate Dilution Well containing the **1 Standard B1 Detection Solution**. 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. Incubate at room temperature for **2 minutes**.

10.6 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 9.1) 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.

10.7 Aspirate the liquid as described above and add **100µl** per well of **TMB Substrate** (pour 1ml per 8 wells in a reservoir). Shake the plate manually for a few seconds and incubate in the dark at room temperature for **3 minutes.**

10.8 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 450 nm as the primary wavelength and optionally 620nm as the reference wave length (610nm to 650nm is acceptable).

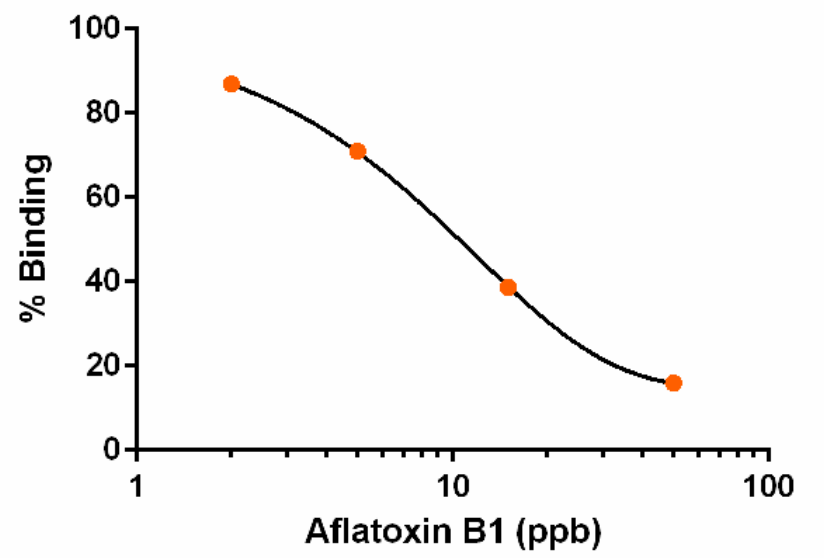
11. Data Analysis

- **Automatically**

An assigned software, the **Prognosis-Data-Reader,** is available for free (contact: info@prognosis-biotech.com) download in order to evaluate the 1 Standard B1 ELISA kit. The evaluation is carried out by a simple transfer of data values after the measurement. B/Bo (%) values of the standards are reported in the Quality Assurance Certificate.

Alternatively, typing the lot number of the kit and the B/Bo (%) values can be automatically downloaded.

12. Example of Standard Curve (0 - 50ppb)



13. Immunoassay Specification

13.1 General Specification

- IC50 = 5.5 - 14 ppb.
- Coefficient of Variation (CV) of result at 5.5 ppb = 5.9% (n=16).
- Coefficient of Variation (CV) of result at 9.5 ppb = 6.4% (n=16).

13.2 LOD - LOQ - Accuracy

- The LOD of the method is 1.5 ppb
- The LOQ of the method is 2.0 ppb.
- The recovery of spiked extractions-matrices was 96.4% (CV = 6.5%).

- **Matrices:**

  Cereals: Barley, Biscuits, Bran Sticks, Brown rice, Corn, Corn flakes, Corn flour, Corn Germ, Corn Gluten (LOQ=3ppb), Corn Grits, Corn Meal, Corn/Soy blend, Dried Sunflower (LOQ=3ppb), Oat bran, Oat flakes, Oats, Popcorn, Raw Rye (LOQ=3ppb), Rice, Rye Flour (LOQ=3ppb), Sesame (LOQ=3ppb), Sorghum, Soy beans (LOQ=3ppb), Wheat, Wheat bran, Wheat flour

  Dried fruit: Dried Figs

  Nuts: Almond, Peanut butter (LOQ=2.5ppb), Raw peanuts, Salted Peanuts

  Other: Milk, Oil (Coconut oil, Corn oil, Olive oil, Olive pomace oil, Peanut oil, Sesame oil, Soybean oil, Sunflower oil)

13.3 Specificity

The cross-reaction of the anti-Aflatoxin B1 antibody with Aflatoxin B1, B2, G1 and G2 is 100, 10, 19 and 1% respectively.

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.

15. Method Summary

Total procedure time (after samples and reagents preparation): 5min

Mix 100µl of the Detection Solution with 100µl of the samples and standard in the Dilution Microwells

⇩

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

⇩

Incubate 2min at room temperature

⇩

Wash four times

⇩

Add 100µl of TMB Substrate

⇩

Let the color develop for 3min in the dark at room temperature

⇩

Add 100µl Stop Solution

⇩

Read Absorbance at 450nm within 60min

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.

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

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