BZERO H2AFLA

Enzyme immunoassay for the detection of total aflatoxins (Cat.nr. HU0040065 / HU0040064)

BZERO H2AFLA is a kit prepared for a competitive immunoenzymatic assay for the quantitative analysis of total aflatoxins.
The kit contains the materials and the procedures sufficient for 96 determinations ***(Cat.nr. HU0040065)*** or 48 determinations ***(Cat.nr. HU0040064)***.
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)
Maize, corn germ, wheat, rice, sorghum, barley, peanut, copra, pistachio, walnut, almond, soybean meal, feed.

Sample preparation
Grinding, extraction with water solution, filtration/centrifugation.

Assay time
10 minutes (sample preparation not included).

Detection limit
2.5 ppb

Specificity	
Compound	**Cross-reactivity %**
Aflatoxin B_1	100
Aflatoxin B_2	93 ± 11
Aflatoxin G_1	86 ± 10
Aflatoxin G_2	76 ± 8

1. TEST PRINCIPLE

The assay is performed in plastic microwells which have been coated with total anti-aflatoxins antibodies In the premixing wells the enzyme conjugate, the standard solution and samples are mixed and then transferred into the anti-aflatoxin microtiter plate.
During the first incubation, free aflatoxins molecules in the standard solution/sample and enzyme-labelled aflatoxins compete for the anti-aflatoxins antibody binding sites on the solid phase.
Any unbound enzyme conjugate and aflatoxins molecules are then removed in a washing step.
The bound enzyme activity is determined adding a fixed amount of colourless chromogenic substrate. The enzyme converts the colourless chromogen into a blue product.
The addition of the stop reagent leads to a colour change from blue to yellow.
The absorbance is measured with a microplate reader at 450 nm. The colour development is inversely proportional to the total aflatoxins concentration in the sample.

2. PROVIDED REAGENTS

Premixing microtiter plate: non-coated wells, blank.
Microtiter plate: coated with total aflatoxins antibody.
As the strips are breakable, the wells can be used individually. For this purpose, it is sufficient to get out the wells from the frame and to break the joint.
Std 0: 1 plastic vials containing 0 ppb of aflatoxin.
ATTENTION: The standard zero is provided only. B/B_0 values of calibration curve (2.5 – 50 ppb) are reported in the kit certificate of analysis.
Enzyme conjugate: plastic bottle.
Extraction buffer 20x: plastic bottle.
Extraction powder bag: bag containing powder for extraction solution preparation.
Washing-buffer 10x: plastic bottle.
Developing solution: plastic bottle.
Stop solution: glass vial. White cap.

Component	(Cat.nr. HU0040065) 96 det.	(Cat.nr. HU0040064) 48 det.
Premixing microplate	96 wells (12 strips x 8 wells)	48 wells (6 strips x 8 wells)
Microtiter plate	96 wells (12 strips x 8 wells)	48 wells (6 strips x 8 wells)
Total aflatoxins Std.	3 ml	1.5 ml
Enzyme conjugate	14 ml	8 ml
Extraction buffer 20x	2 x 62 ml	1 x 62 ml
Extraction powder bag	4 bags	2 bags
Washing buffer 10x	50 ml	50 ml
Developing solution	14 ml	8 ml
Stop solution	8 ml	6 ml

3. REQUIRED BUT NOT PROVIDED MATERIALS

- Distilled water

Equipment
- Balance
- For grinding: grinder or blender (like “Osterizer”).
- For extraction (optional): shaker
- Filter paper (Whatman 1); optional: centrifuge
- 20-200 µl micropipette, tips
- 100-1000 µl micropipette, tips
- 50-300 µl multichannel micropipette, tips

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- Laboratory glassware (beaker, graduated cylinder, bottle)
- Microtiter plate, filter 450 nm.

4. WARNING AND PRECAUTIONS FOR THE USERS

- The test for *in vitro* diagnostic use only.
- Some reagents contain solutions that may be identified as dangerous substance by the Regulation (EC) *No 1272/2008. Please refer to Material Safety Data Sheet available on both the Eurofins Technologies (eurofins-technologies.com) 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

ATTENTION:
- **Equilibrate the extraction solution at room temperature (20-25°C) before use.**
- **Analyze the sample in the test immediately after the extraction. Do not store the extract for further analyses.**
- **For filtration use only polypropylene vials.**

6.1 Maize, wheat, rice, sorghum, barley, copra

1) Mix carefully the sample to be analysed in order to make it homogeneous.
2) Finely grind the sample.
3) Weigh 5 g of the sample.
4) Add 25 ml of **extraction solution**.
5) Shake vigorously for 3 minutes.
6) Filter the sample (Whatman 1) and collect the filtrate.
7) If the sample occurs to be contaminated at a concentration >50 ppb, dilute the filtrate 5 times (1+4) in the extraction solution in order to obtain a dosage range of 12.5-250 ppb (i.e.: 100 µl of filtered sample + 400 µl of extraction solution).

6.2 Peanut, soybean meal, corn germ, feed

1) Mix carefully the sample to be analysed in order to make it homogeneous.
2) Finely grind the sample.
3) Weigh 5 g of the sample.
4) Add 25 ml of **extraction solution**.
5) Shake vigorously for 3 minutes.
6) Filter the sample (Whatman 1) and collect the filtrate.
7) **ATTENTION:** The time of filtration must not exceed 30 minutes. In case of difficult filtration, it is possible to centrifuge the extract: take 1 ml of extract and centrifuge 1 minute at 3500 x g. Collect the supernatant, avoiding the upper fat layer eventually present.
8) If the sample occurs to be contaminated at a concentration >50 ppb, dilute the extract 5 times (1+4) in the extraction solution in order to obtain a dosage range of 12.5-250 ppb (i.e.: 100 µl of filtered sample + 400 µl of extraction solution).

6.3 Pistachio, walnut

1) Mix carefully the sample to be analysed in order to make it homogeneous.
2) Finely grind the sample.
3) Weigh 5 g of the sample.
4) Add 25 ml of **2x extraction solution**.
5) Shake vigorously for 3 minutes.
6) Filter the sample (Whatman 1) and collect the filtrate.
7) **ATTENTION:** The time of filtration must not exceed 30 minutes. In case of difficult filtration, it is possible to centrifuge the extract: take 1 ml of extract and centrifuge 1 minute at 3500 x g. Collect the supernatant, avoiding the upper fat layer eventually present.
8) If the sample occurs to be contaminated at a concentration >50 ppb, dilute the filtrate 5 times (1+4) in the 2x extraction solution in order to obtain a dosage range of 12.5-250 ppb (i.e.: 100µl of filtered sample + 400µl of 2x extraction solution).

6.4 Almond

1) Mix carefully the sample to be analysed in order to make it homogeneous.
2) Finely grind the sample.
3) Weigh 5 g of the sample.
4) Add 25 ml of **2x extraction solution**.
5) Shake vigorously for 3 minutes.

6) Filter the sample (Whatman 1) and collect the filtrate.
7) If the sample occurs to be contaminated at a concentration >50 ppb, dilute the filtrate 5 times (1+4) in the 2x extraction solution in order to obtain a dosage range of 12.5-250 ppb (i.e.: 100µl of filtered sample + 400µl of 2x extraction solution)

7. WORKING SOLUTIONS PREPARATION

Std 0: ready to use. Shake prior to use.
Enzyme conjugate: ready to use.

Extraction solution. To prepare 600 ml of extraction solution:
1) Pour the content of one "Extraction powder bag" into a suitable container.
2) Rinse the bag with about 50ml of distilled water, and transfer the liquid inside the container.
3) Add in the container about 450ml of distilled water and mix until the powder is completely dissolved.
4) Add 30 ml of Extraction Buffer 20x and mix. **ATTENTION:** In presence of crystals, bring the solution at room temperature and mix in order to solve them completely.
5) Using a cylinder, bring to 600 ml volume with distilled water and mix thoroughly.

Extraction solution is stable all kit shelf life long at +2/+8 °C, when stored in a tightly closed container.

2x Extraction solution. To prepare 300 ml of extraction solution:
1) Pour the content of one "Extraction powder bag" into a suitable container.
2) Rinse the bag with about 50ml of distilled water, and transfer the liquid inside the container.
3) Add in the container about 150ml of distilled water and mix until the powder is completely dissolved.
4) Add 30 ml of Extraction Buffer 20x and mix. **ATTENTION:** In presence of crystals, bring the solution at room temperature and mix in order to solve them completely.
5) Using a cylinder, bring to 300 ml volume with distilled water and mix thoroughly.

2x Extraction solution is stable all kit shelf life long at +2/+8 °C, when stored in a tightly closed container.

Washing buffer: dilute the concentrate 1:10 (1+9) with distilled water; **ATTENTION:** In presence of crystals, bring the solution at 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; this solution is light sensitive, keep away from direct light.
Stop solution: ready to use. **ATTENTION:** It contains 1M sulphuric acid. Handle with care; in case of contact flush immediately with plenty of water.

8. ASSAY PROCEDURE

1) Predispose the assay layout, recording standard solution and samples positions, taking into account that one well is required for each standard and sample. Prepare an equal number of premixing wells. **ATTENTION:** It is suggested to carry out no more than 48 determinations in each assay (standards included);
if a multichannel pipette is not used, it is advised to carry out no more than 16 determinations in each assay (standards included).
2) First incubation:
   - Add 100 µl of enzyme conjugate **in each premixing well**.
   - Add 50 µl of standard 0 and each sample into the corresponding premixing wells and mix the content of the well by pipetting up and down three times.
   - Transfer 100 µl from the premixing wells into the corresponding anti-aflatoxins antibody coated microwells. Do not pipette the solution during this phase.
   - **ATTENTION:** use new tips for each well to avoid cross-contamination.
   - Incubate 5 minutes at room temperature;
   - *Do not prolong the first incubation time and do not shake during incubation.*
3) Washing sequence
   - Pour the liquid out from the wells.
   - Fill completely all the wells with washing buffer 1x 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) Developing
   - Add 100 µl of developing solution to each well.
   - Mix thoroughly with rotatory motion for few seconds.
   - Incubate for 5 minutes at room temperature. Protect from light.
5) Add 50 µl of stop solution to each well and mix thoroughly with rotatory motion for few seconds.
6) Measure the absorbance at 450 nm.
7) Read within 15 minutes.

9. RESULTS CALCULATION

- Divide the absorbance value of each sample by the absorbance of the Standard 0 (B_0) and multiply by 100; the standard 0 (B_0) is thus made equal to 100% and all the other absorbance values are expressed as percentage:

$$\frac{\text{Standard (or sample) absorbance}}{\text{Standard 0 } (B_0) \text{ absorbance}} \times 100 = \frac{B}{B_0}(\%)$$

- Enter the B/B0 values provided for each standard (0.2; 0.5; 10; 50 ppb) in the kit certificate of analysis in a semi-logarithmic system of coordinates against the aflatoxins standard concentration and draw the standard curve.
- Interpolate the B/B_0 value for each sample it to the corresponding concentration from the calibration curve.
- Standards aflatoxins concentration (ppb) already consider the sample dilution factor.
- If the sample was 1:5 furtherly diluted in order to obtain a dosage range 12.5-250 ppb, multiply by a factor 5 the obtained concentration value.

Please note: for results calculation it is suggest to use the "point to point" 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.

11. EVALUATION OF RESULTS

After processing the results and creating the calibration curve, it is necessary to verify the assay performance. The verification is performed by comparison of obtained data with those given in kit specifications (chapter 12).
If the values are out of specifications, then the results of the test are not assured, therefore the aflatoxins 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.

12. KIT SPECIFICATIONS

12.1 Assay specification

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

12.2 Assay performance

Matrix	Cut off (ppb)	LOQ (ppb)
Maize	3	4
Corn germ	4	7.5
Wheat	$\leq$ 2.5	4
Rice	$\leq$ 2.5	4
Sorghum	3.7	ND
Barley	4	7.5
Peanut	2.8	4
Copra	$\leq$ 2.5	5
Pistachio	4.8	5
Walnut	3	5
Almond	3	7.5
Soybean meal	5	10
Bovine feed	7	8
Feed for other animals	7	10

ND: not determined

13. 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.