Celer AFLA B_1

Enzyme immunoassay for the detection of Aflatoxin B_1 (Cat. nr. HU0040004)

Celer AFLA B₁ is a kit prepared for a competitive immunoenzymatic assay for the quantitative analysis of aflatoxin B_1.
The kit contains the procedure and the materials sufficient for 96 determinations including standards.
For result evaluation a microtiter plate or strip photometer is required (manual or automatic ELISA reader).

Type of samples that can be analyzed (matrices)
Cereals (sorghum, corn, high moisture corn, brown rice), corn gluten meal, silage and mash, feed, nuts (hazelnuts, pistachios, peanuts, almonds), peanut meal, dried fruits (raisins, figs), DDGS, cottonseed, soy, soybean meal.

Sample preparation
- Cereals, high moisture corn, brown rice, corn gluten meal, silage and mash, feed, nuts, peanut meal, dried fruits, DDGS, soy and soybean meal: grinding and homogenization, extraction in methanol-water, filtration
- Cottonseed: grinding and homogenization, extraction in methanol-water, sedimentation.

Assay time: 15 minutes (sample preparation not included).

Detection limit
1 ppb

Specificity

Compound	Cross-reactivity %
Aflatoxin B_1	100
Aflatoxin B_2	5 ± 1
Aflatoxin G_1	19 ± 1
Aflatoxin G_2	<1

1. TEST PRINCIPLE

The assay is performed in plastic microwells that have been coated with anti-aflatoxin antibody. In the premixing wells the enzyme labelled aflatoxin and the standard solutions or samples are mixed and then transferred into the anti-aflatoxin microtiter plate. During the first incubation, free aflatoxin in the standard solution /sample and enzyme-labelled aflatoxin compete for the anti-aflatoxin antibody binding sites on the solid phase. Any unbound enzyme conjugate and aflatoxin molecule are then removed in a washing step. The bound enzyme activity is determined adding a fixed amount of a 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 aflatoxin B_1 concentration in the standard solution /sample.

2. PROVIDED REAGENTS

Premixing microtiter plate: 96 wells (12 strips of 8 wells) non-coated wells, blank.
Microtiter plate: 96 wells (12 strips of 8 wells) coated with anti-aflatoxin 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.
Aflatoxin B_1 std: 5 plastic vials containing 1.5 ml of: 0 ppb; 1 ppb; 5 ppb; 20 ppb; 40 ppb of aflatoxin B_1.
Enzyme conjugate: 1 plastic bottle containing 14 ml.
Washing-buffer 10x: 1 plastic bottle containing 50 ml.
Developing solution: 1 plastic bottle containing 14 ml.
Stop solution: 1 glass vial containing 8 ml. White cap.

3. REQUIRED BUT NOT PROVIDED MATERIALS

- Distilled water
- Methanol
- 80% Methanol (dried fruits, peanut meal); Mycotoxin Extraction Solution A" Cat. nr. HU0040103 or 70% methanol (cereals, feed, high moisture corn, silage and mash, DDGS, cottonseed, corn gluten meal); 60% methanol (nuts, soy, soybean meal, brown rice).
- NaCl (for cereals, high moisture corn, brown rice, silage and mash, feed, nuts, peanut meal, dried fruits, cottonseed, soy, soybean meal).

Equipment
- Balance
- For grinding: grinder or blender (like "Osterizer").
- For extraction (optional): shaker
- Filter paper (Whatman 1)
- Centrifuge (optional, for cottonseed)
- 20-200 ul micropipette, tips
- 50-300 ul multichannel micropipette, tips
- 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 substances by the Regulation (EC) No 1272/2008. Material Safety Data Sheets are available on the Eurofins Technologies and Eurofins Tecna *(www.tecna.eurofins-technologies.com*) websites.
- 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.

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- 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: The extracts can only be used within the day of extraction. Do not store it for longer time. It is suggested to weigh 50 gr in order to have a more representative analysis of the sample. Particularly for cottonseed, do not extract less than 50 gr of sample.

6.1 Cereals and feed

- Mix carefully the sample to be analysed in order to make it homogeneous.
- Finely grind the sample.
- Weigh the sample, choosing among the options described in the following table

Sample	NaCl	Extraction solution
50 g	10 g	250 ml 70% methanol
5 g	1 g	25 ml 70% methanol
50 g	/	250 ml 70% methanol, 4% NaCl*
5 g	/	25 ml 70% methanol, 4% NaCl*

*** Preparation of extraction solution with 70% methanol and 4% NaCl:**
For 100 ml of solution: dissolve 4 g of NaCl in 20 ml of deionized or distilled water, add 70 ml of methanol, then add deionized or distilled water to 100 ml.

- Shake thoroughly for 3 minutes.
- Filter the sample (Whatman 1) and collect the filtrate.

If the sample is dosed >40 ppb, dilute the extract 5 times (1+4) in methanol 70%, in order to obtain a dosage range 5-200 ppb.

6.1.1 High moisture corn

- Extract samples according to point 6.1 procedure.
- To relate result to dry matter, take into account the sample moisture percentage.

6.1.2 Silage and mash

- Extract samples according to point 6.1 procedure. Adjust extracts pH to 6.5-7.5 with 5M NaOH.
- If dry samples are analysed, it is suggested to dry them at temperatures not higher than 60°C.

6.2 Nuts, soy, soybean meal, brown rice

- Mix carefully the sample to be analysed in order to make it homogeneous.
- Finely grind the sample.
- Weigh 50 g of ground sample and add 10 g of NaCl. Add 250 ml of a solution of 60% methanol in distilled water. **Alternatively**: weigh 5 g of ground sample and add 1 g of NaCl. Add 25 ml of a solution of 60% methanol in distilled water. **ATTENTION:** it is possible to prepare an extraction solution which already contains NaCl. For 100 ml of solution: dissolve 4 gr of NaCl in 20 ml of deionized or distilled water, add 60 ml of methanol, then add deionized or distilled water to 100 ml.
- Shake thoroughly for 3 minutes.
- Filter the sample (Whatman 1) and collect the filtrate.

6.3 Dried fruits, peanut meal

- Finely mince the sample (dried fruit).
- Weigh 5 g of minced sample.
- Add 0,5 g of NaCl.
- Add 25 ml of a solution of MeOH 80% in distilled water. **ATTENTION:** it is possible to prepare an extraction solution which already contains NaCl. For 100 ml of solution: dissolve 2 g of NaCl in 15 ml of deionized or distilled water, add 80 ml of methanol, then add deionized or distilled water to 100 ml.
- Shake thoroughly for 3 minutes.
- Filtrate the sample (Whatman 1) or centrifuge at 3500xg for 5'; recover the supernatant /filtrate.

6.4 DDGS

- Mix carefully the sample to be analysed in order to make it homogeneous.
- Finely grind the sample.
- Weigh 50 g of sample and add 250 ml of a solution of 70% methanol in distilled water. **Alternatively:** weigh 5 g of sample and add 25 ml of a solution of 70% methanol in distilled water.
- Shake thoroughly for 15 minutes.
- Filter the sample (Whatman 1) and collect the filtrate.
- If the sample is dosed >40 ppb, dilute the extract 5 times (1+4) in methanol 70%, in order to obtain a dosage range 5-200 ppb.

6.5 Cottonseed

- Mix carefully the sample to be analysed in order to make it homogeneous.
- Finely grind the sample.
- Weigh the sample, choosing among the options described in the following table

Sample	NaCl	Extraction solution
50 g	10 g	250 ml 70% methanol
50 g	/	250 ml 70% methanol, 4% NaCl*

***Preparation of extraction solution with 70% methanol and 4% NaCl:**

For 100 ml of solution: dissolve 4 g of NaCl in 20 ml of deionized or distilled water, add 70 ml of methanol, then add deionized or distilled water to 100 ml.

- Shake thoroughly for 3 minutes.
- Let the sample settle for phase separation, then collect the supernatant. As alternative, for an easier separation, centrifuge the sample at 3500 g for 5 minutes.
- If the sample is dosed >40 ppb, it is suggested to dilute the extract 1:5 with 70% methanol and to repeat analysis. A lower dosage of some samples in respect to the first analysis is due to a lower matrix effect as a consequence of extract dilution.

6.6 Corn gluten meal

- Weigh 50 g of ground sample and add 250 ml of a solution of 70% methanol in distilled water. **Alternatively:** weigh 5 g of ground sample and add 25 ml of a solution of 70% methanol in distilled water.
- Shake thoroughly for 3 minutes.
- Filter the sample (Whatman 1) and collect the filtrate.

7. WORKING SOLUTIONS PREPARATION

Aflatoxin B_1 std: ready to use; mix before use.
Enzyme conjugate: ready to use.
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 1 M sulphuric acid. Handle with care and in case of contact flush immediately with plenty of water.

8. ASSAY PROCEDURE

1) Predispose the assay layout, recording standard solutions 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 each standard/ sample into the corresponding premixing wells. The standard/sample contain high percentage of methanol: take care to rinse the tip pipetting up and down the solutions before adding to the wells.
   - Using the micropipette, mix the content of each premixing well (pipette up and down three times) and immediately transfer 100µl into the corresponding anti-aflatoxin B1 antibody coated microwell.
   - **ATTENTION**: use new tips for each well to avoid cross-contamination.
   - Incubate 10 minutes at room temperature;
   - Do not prolong the first incubation time and do not use automatic shakers.
3) Washing
   - 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 direct 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. CALCULATION OF RESULTS

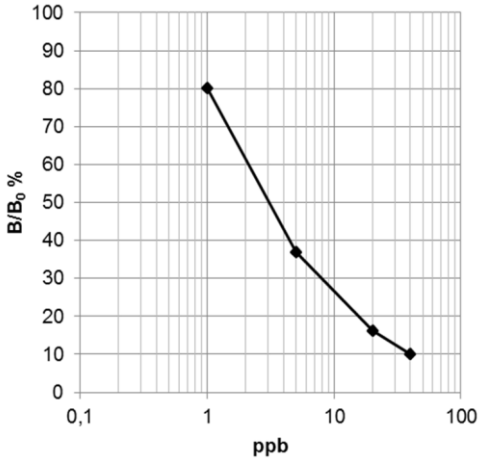
- Divide the absorbance value of each standard and sample by the absorbance of the standard 0 (B_0) and multiply by 100; the standard 0 (B_0) is thus made equal to 100% and all the other absorbance values are expressed as percentage:

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

- Enter the B/B_0 values calculated for each standard in a semi-logarithmic system of coordinates against the aflatoxin B1 standard concentration and draw the standard curve.
- Interpolate the B/B_0 value of each sample to the corresponding concentration from the calibration curve. Standards concentration (ppb) already considers the sample dilution factor. If the extract was 5 fold diluted, multiply by 5 the obtained concentration value.

Please note: to build the calibration curve, the "spline" algorithm can be used, but it is also possible to use the "linear regression" or the "point to point" curve. To elaborate the ELISA results using the "point to point" method, Excel spreadsheet are available. Please contact the technical assistance.

10. CALIBRATION CURVE EXAMPLE



11. EVALUATION OF RESULTS

After processing the results, it is necessary to verify the assay performance. The verification is performed by comparison of obtained data with those given in kit specifications (see chapter 12).
If the values are outside the specifications given, then the results of the test are not assured, therefore the aflatoxin B1 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}
B/B_0 50%	1.6 – 8 ppb

12.2 Assay performance

Matrix	Concentration ppb	Recovery % ± ds
Corn (CRM)	2-90	92 ± 17
Sorghum (spiked)	2.5-15	80 ± 8
Corn germ (incurred)	10-20	79 ± 8
Huzelnut (incurred)	10	73 ± 4
Almond (spiked)	2.5-15	100±10
Peanuts (spiked)	2.5-15	113 ± 8
Raisins (spiked)	5-10	78 ± 10
Soy (spiked)	2.5-15	97 ± 6
Soybean meal (spiked)	10-30	106 ± 10
Brown rice (RM)	16-29	102 + 19
Corn gluten meal (RM)	5-31	83 + 14

The results were obtained by means of a "4 parameters" and point to point elaboration of the calibration curve.

Matrix	Cut off - ppb	LOQ- ppb
Maize	$\leq$ 1	1
Sorghum	$\leq$ 1	ND
Nuts	$\leq$ 1	ND
Dried fruits	$\leq$ 1	2
Soybean meal	2.5	5

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.

14. REFERENCES

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