Celer AFLA

Enzyme immunoassay for the detection of total aflatoxins (Cat.nr. HU0040011 / HU0040031)

Celer AFLA is a kit prepared for a competitive immunoenzymatic assay for the quantitative analysis of total aflatoxins.
The kit contains the procedure and the materials sufficient for 96 determinations **(Cat.nr. HU0040011)** or 48 determinations **(Cat.nr. HU0040031)** 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, corn germ, feed, cottonseed, nuts, dried fruits, paprika, chilli, ginger.

Sample preparation

- Cereals, nuts, dried fruits, ginger: grinding and homogenization, extraction in methanol-water, filtration
- Corn germ, feed, cottonseed: grinding and homogenization, extraction in methanol-water, filtration, purification on EasypurAFLA.
- Paprika, chilli: grinding and homogenization, extraction in methanol-water, filtration, dilution, filtration, purification on affinity column, evaporation, resuspension.

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

Detection limit
2 ppb

1. TEST PRINCIPLE

The assay is performed in plastic microwells that have been coated with anti- aflatoxins antibody. In the premixing wells the enzyme conjugate and the standard solutions or samples are mixed and then transferred into the anti- aflatoxins microtiter plate.
During the first incubation, free aflatoxins in the standard solution /sample and enzyme-labelled aflatoxin compete for the anti- aflatoxins antibody binding sites on the solid phase. Any unbound enzyme conjugate and f aflatoxins 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 total aflatoxins concentration in the standard solution /sample.

2. PROVIDED REAGENTS

Premixing microtiter plate: microplate non-coated wells, blank.
Microtiter plate: microplate coated with anti- 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.
Total Aflatoxins std: 5 plastic vials containing the total aflatoxins solutions at: 0 ppb; 2 ppb; 8 ppb; 30 ppb; 80 ppb.
Enzyme conjugate: 1 plastic bottle.
Washing-buffer 10x: 1 plastic bottle.
Developing solution: 1 plastic bottle.
Stop solution: 1 glass vial. White cap.

Component	Cat.nr. HU0040011 96 det.	Cat.nr. HU0040031 48 det.
Premixing microplate	96 wells (12 strips x 8 wells)	48 wells (6 strip x 8 wells)
Microtiter plate	96 wells (12 strips x 8 wells)	48 wells (6 strips x 8 wells)
Total Aflatoxins Std.	5 vial x 1.5 ml	5 vial x 1.5 ml
Enzyme conjugate	14 ml	8 ml
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.
- Methanol (80% Methanol for dried fruits, paprika and chilli); 70% methanol for cereals, ginger, feed, corn germ, cottonseed; 60% methanol for nuts)
- NaCl.
- n-hexane and PBS (pH 7,2; for paprika and chilli).

Equipment

- Balance.
- For grinding: grinder or blender (like “Osterizer”).
- For extraction (optional): shaker
- Filter paper (Whatman 1).
- EasypurAFLA (Cat.nr. AC010; for corn germ, feed, cottonseed)
- Centrifuge (optional, for corn germ, feed, cottonseed)
- Glassfiber filter (Whatmann 934-AH for paprika/chilli).
- 20-200 µl micropipette, tips
- 100-1000 µl micropipette, tips
- 50-300 µl multichannel micropipette, tips
- Microtiter plate, filter 450 nm.

Eurofins Technologies Hungary Kft., Fóti út 56., 1047 Budapest, Hungary
E-mail: technologies.hu@eurofins.com
Website: www.eurofins-technologies.com

4. WARNING AND PRECAUTIONS FOR THE USERS

- The test is for *in vitro* diagnostic use only.
- Some reagents contain solutions that may be identified as dangerous substance by the Regulation (EC) *N° 1272/2008*. Please refer to *Material Safety Data Sheet* available on both the Eurofins Technologies 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 (RT= 18 – 25 °C) 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: 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, ginger

1) Mix carefully the sample to be analysed in order to make it homogeneous.
2) Finely grind the sample.
3) Weigh the sample, choosing among the options described in the following table:

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

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

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

6.2 Corn germ, feed, cottonseed

1) Mix carefully the sample to be analysed in order to make it homogeneous.
2) Finely grind the sample.
3) 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**

***: not suggested for cottonseed**

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

4) Shake thoroughly for 3 minutes.
5) Filter the sample (Whatman 1) and collect the filtrate. **ATTENTION:** For cottonseed, if filtration turns out to be difficult, let the sample settle and take the supernatant.
6) Add 2 ml of filtrate/supernatant to an *EasypurAFLA* tube (Cat.nr.AC010).
7) Mix vigorously for 30 seconds. Take care to resuspend the gel completely.
8) Centrifuge 5 minutes at 1500xg or let the gel settle for 10 minutes.
9) The clear supernatant is ready for analysis; it is suggested to transfer it into a 1.5 ml tube in order to avoid the gel resuspension during the transferring of the extract into the microtiter plate.

6.3 Nuts

1) Mix carefully the sample to be analysed in order to make it homogeneous.
2) Finely grind the sample.
3) 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.
4) Shake thoroughly for 3 minutes.
5) Filter the sample (Whatman 1) and collect the filtrate.

6.4 Dried fruits

1) Mix carefully the sample to be analysed in order to make it homogeneous.
2) Finely grind the sample.
3) Weigh 50 g of ground sample

4) Add 5 g of NaCl.
5) Add 250 ml of a solution of 80% methanol in distilled water.

Alternatively: Weigh 5 g of ground sample and add 0.5 g of NaCl. Add 25 ml of a solution of 80% methanol in distilled water.

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

6.5 Paprika and chilli

ATTENTION: In case of whole chilli finely grin the sample.

1) Weigh 5 g of sample.
2) Add 0.5 g of NaCl.
3) Add 25 ml of methanol 80%
4) Add 12.5 ml of n-hexane.
5) Shake thoroughly for 3 minutes.
6) Filter on filter (Whatman 1).
7) Rescue about 3 or 4 ml of the lower methanolic phase.
8) To 2.8ml of the methanolic phase add 17.2 ml of PBS 1X (pH 7.2).
9) Filter on glassfiber filter (Whatmann 934-AH).

Column

ATTENTION: Before use, bring the immunoaffinity columns to room temperature.

10) Eliminate the storage solution from the column.
11) Add 10 ml of the extract (2 ml/min or gravity).
12) Wash the column with 10ml of PBS1X.
13) Remove the PBS residue with vacuum.
14) Elute with methanol 100% (1 ml for 2 times)
15) Rescue completely the methanol form the column.
16) Evaporate the eluate at 40°C and resuspend with 1.4 ml of methanol 80%.

7. WORKING SOLUTIONS PREPARATION

Total Aflatoxins 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 100 µl into the corresponding anti-aflatoxins 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 60 minutes.

9. RESULTS CALCULATION

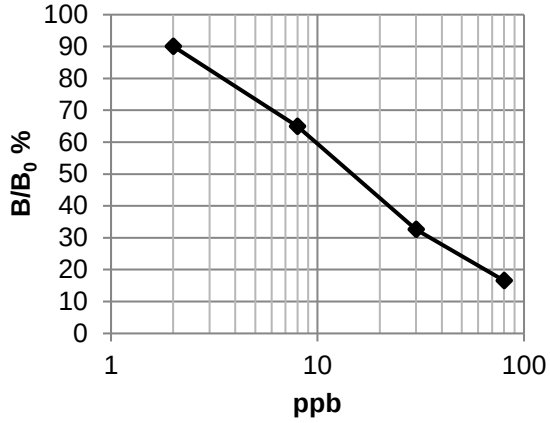
– Divide the absorbance value of each standard and sample by the absorbance of the standard 0 (B_0) and multiply by 100; the standard 0 (B_0) is thus made equal to 100% and all the other absorbance values are expressed as percentage:

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

- Enter the B/B_0 values calculated for each standard in a semi-logarithmic system of coordinates 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.

Please note: to build the calibration curve, the "spline" algorithm can be used, but it is also possible to use the "point to point" curve or the "linear regression".
To elaborate the ELISA results using the "point to point" method, Excel spreadsheet are available on the Eurofins Tecna website and can be downloaded directly from the bottom of the product page.

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 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}
B/B_0 50%	6-25 ppb

12.2 Assay performance

Matrix	Cut off ppb	LOQ ppb
Maize	3	2
Corn germ	ND	ND
Feed	ND	ND
Cottonseed	ND	ND
Ginger	ND	ND
Paprika/chilli	$\leq$ 2	5
Huzelnuts	3.4	5
Pistachio	2.6	5
Figs and raisins	$\leq$ 2	5

ND: not determined

Matrix	Concentration ppb	Recoveries % $\pm$ ds
Corn (spiked)	$\leq$ 4	134 $\pm$ 19
	8-20	94 $\pm$ 12
	40-80	77 $\pm$ 7
Corn germ (incurred)	10-25	105 $\pm$ 10
Feed (CRM)	15-30	90 $\pm$ 17
Paprika/chilli	5	97 $\pm$ 25
Huzelnuts (CRM)	3.7	114 $\pm$ 14
Pistachio	5	84 $\pm$ 10
Figs and raisins	5	80 $\pm$ 15
	7.5	77 $\pm$ 15

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

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. LITERATURE

Kirilov, I. M., Đokić, G. M., & Popov, S. Z. (2013). Validation of immunoenzymatic tests for the detection of aflatoxin present in food. Jour. Nat. Sci, Matica Srpska Novi Sad (124), 37-50.

G. Rosar, L. Persic, F. Gon, B. Puppini, V. Bassani, F. Diana. Analysis of mycotoxins in complex matrices by enzyme immunoassays. Poster presentation at 35th Mycotoxin Workshop, 2013, 22-24 May, Ghent, Belgium.

F. Gon, G. Rosar, E. Paoluzzi, F. Diana. Mycotoxin poly-contamination in maize: fast and sensitive ELISA test kits for a multi-analytical screening. Poster presentation at RME 2013 21-23 January, Noordwijkerhout, the Netherlands.

Diana, F., Persic, L., Tamburlini, F., and Paleologo, M. Celer AFLA: a rapid enzyme immunoassay for the quantitative determination of total aflatoxins in cereals and feed. Poster presentation at the 13th ICC Cereal and Bread Congress. International Association for Cereal Science and Technology. June 15-18, 2008. Madrid, Spain.