INSTRUCTION FOR USE

Celer OCHRA

(Cat. nr. HU0040013)

Enzyme immunoassay for the detection of ochratoxin A

Celer OCHRA is a kit prepared for a competitive immunoenzymatic assay for the quantitative analysis of ochratoxin A.
The kit contains the procedure and the materials sufficient for 96 determinations including standards.
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)
Cereals, wheat bran, buckwheat, buckwheat flour, cocoa beans and cocoa powder (natural or alkalized sample).

Sample preparation
Cereals, wheat bran, buckwheat, buckwheat flour: grinding, extraction in methanol-water, filtration.
cocoa beans and cocoa powder: grinding, extraction with solvent, purification with IAC, dilution.

Assay time: 20 minutes (sample preparation not included).

Detection limit
Cereal: 2 ppb.
Wheat bran: 4 ppb
Buckwheat: 2 ppb
Buckwheat flour: 2 ppb
Cocoa beans: 2 ppb
Cocoa powder: 2 ppb

1. TEST PRINCIPLE

The assay is performed in plastic microwells that have been coated with anti- ochratoxin antibody. In the premixing wells the enzyme labelled ochratoxin and the standard solutions or samples are mixed and then transferred into the anti- ochratoxin microtiter plate. During the first incubation, free ochratoxin in the standard solution /sample and enzyme-labelled aflatoxin compete for the anti-ochratoxin antibody binding sites on the solid phase. Any unbound enzyme conjugate and ochratoxin 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 ochratoxin 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-ochratoxin 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.
Ochratoxin A std: 5 glass vials containing 1.5 ml of ochratoxin A solution in the following concentrations: 0 ppb; 2 ppb; 5 ppb; 25 ppb; 50 ppb.
Enzyme conjugate: 1 glass vial containing 200 µl concentrated enzyme conjugate.
Enzyme conjugate diluent: 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

- NaCl
- Distilled water
- Methanol or "Mycotoxin Extraction Solution A" Cat. nr. HU0040103
- Hydrochloric acid (HCl) 1M – only for Cocoa samples
- Sodium bicarbonate solution 2% – only for Cocoa samples
- PBS 1X – only for Cocoa samples
- SENSIColumn IAC Ochratoxin SMART (EFOTS3125) – only for Cocoa samples

Equipment
- Balance
- For grinding: grinder or blender (like "Osterizer").
- For extraction (optional): shaker
- Filter paper (Whatman 1)
- 20-200 µl micropipette, tips
- 100-1000 µl micropipette, tips
- 50-300 µl 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 substance by the Regulation (EC) *No 1272/2008. Please refer to Material Safety Data Sheet available on Gold Standard Diagnostics web site: www.goldstandarddiagnostics.com.*
- 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.
- **ATTENTION**: For sample preparation it is advised to use glass vials.

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 g in order to have a better representative analysis of the sample.

6.1 Cereals, buckwheat and buckwheat flour

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 extraction solution 70% methanol, 4% NaCl (*)
5 g	/	25 ml extraction solution 70% methanol, 4% NaCl (*)

(*) 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) The filtrate is ready for the assay.

6.2 Wheat bran

1) Mix carefully the sample to be analysed in order to make it homogeneous.
2) Finely grind the sample.
3) Weigh 5 g of sample and add 50 ml of 70% methanol
4) Shake thoroughly for 3 minutes.
5) Filter the sample (Whatman 1) and collect the filtrate.
6) The filtrate is ready for the assay.
7) The dilution factor is 2.

6.3 Cocoa beans and natural cocoa powder

1) Mix carefully the sample to be analyzed in order to make it homogeneous.
2) Finely grind the sample.
3) Weigh 10 g of sample and add 40 ml of methanol 100%.
4) Shake thoroughly for 3 minutes.
5) Add 40 ml of sodium bicarbonate solution 2%.
6) Shake thoroughly for 3 minutes.
7) Filter the sample (Whatman 1)
8) Dilute 8 ml of filtrate with 22 ml of PBS1X.
9) Check and adjust the pH at 7.4 with HCl 1M.

Column

10) Eliminate the storage buffer from the column.
11) Load 30 ml of diluted extract (2 mL/min or gravity)
12) Wash the column with 30 ml of PBS1X.
13) Remove the PBS 1X with the vacuum.
14) Put the cap to column and add 1 ml of methanol 100% and wait 1 minute before the elution. Repeat 1 ml for 2 times. (Total cycles 2)
15) Rescue completely the methanol from the column.
16) Diluted the eluate 1:2.5X with methanol 70% (ex. 100 µl of eluate + 150 µl of methanol 70%).

6.4 Alkalized cocoa powder (high and low fat)

1) Mix carefully the sample to be analyzed in order to make it homogeneous.
2) Finely grind the sample.
3) Weigh 10 g of sample and add 40 ml of methanol 50% and NaCl 4%.
4) Shake thoroughly for 3 minutes.
5) Add 40 ml of sodium bicarbonate solution 2%.
6) Shake thoroughly for 3 minutes.
7) Filter the sample (Whatman 1)
8) Dilute 8 ml of filtrate with 22 ml of PBS1X.
9) Check and adjust the pH at 7.4 with HCl 1M.

Column

10) Eliminate the storage buffer from the column.
11) Load 30 ml of diluted extract (2 mL/min or gravity)
12) Wash the column with 30 ml of PBS1X.
13) Remove the PBS 1X with the vacuum.
14) Put the cap to column and add 1 ml of methanol 100% and wait 1 minute before the elution. Repeat 1 ml for 2 times. (Total cycles 2)
15) Rescue completely the methanol from the column.
16) Diluted the eluate 1:2.5X with methanol 70% (ex. 100 µl of eluate + 150 µl of methanol 70%).

7. WORKING SOLUTIONS PREPARATION

Ochratoxin standards: ready to use; mix before use.
Enzyme conjugate diluent: ready to use.
Enzyme conjugate: calculate and prepare the quantity necessary for the experiment. Dilute the conjugate **1/200** with the enzyme diluent.
ATTENTION: In order not to take less than 20 µl of concentrated enzyme conjugate make two consecutive dilutions.
For example: prepare a semiconcentrated **1/10** (take 50 µl of concentrated enzyme coniugate + 450 µl of enzyme diluent) and prepare the ready-to-use coniugated by diluting the semiconcentrated **1/20 (**take 150 µl of semiconcentrated coniugate + 2850 µl of enzyme diluent).
DO NOT VORTEX.
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-ochratoxin 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 10 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. RESULTS CALCULATION

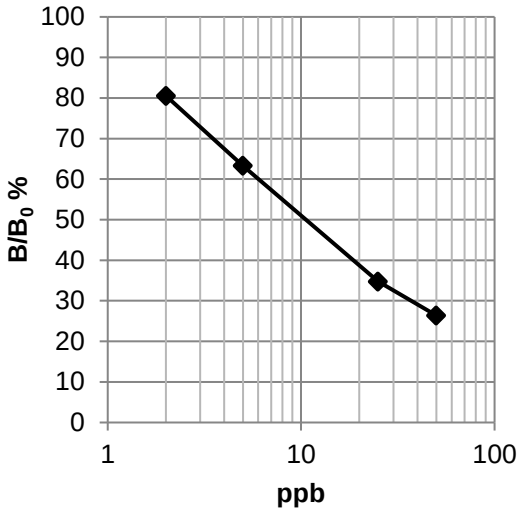
- Divide the absorbance value of each standard and sample by the absorbance of the standard 0 (B0) and multiply by 100; the standard 0 (B0) is thus made equal to 100% and all the other absorbance values are expressed as percentage:

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

- Enter the B/B_0 values calculated for each standard in a semi-logarithmic system of coordinates against the ochratoxin standard concentration and draw the standard curve.
- Take the B/B_0 value for each sample and interpolate it to the corresponding concentration in the calibration curve. Standards concentration (ppb) already considers the sample dilution factor for cereal samples. For wheat bran, multiply by 2 the obtained result.

Please note: for results calculation it is suggest to use the "point to point" curve. Excel spreadsheet are available in the Document Repository section of the Gold Standard Diagnostics web site (www.goldstandarddiagnostics.com).

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 ochratoxin 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%	5.5 - 19 ppb

12.2 Assay performance

Matrix	Cut off (ppb)	LOQ (ppb)
Barley	3.5	5
Wheat	3	4
Maize	3.5	5
Wheat bran	≤4	5
Buckwheat	2.2	2.5
Buckwheat flour	3.5	5
Cocoa beans	≤2	2

Matrix	Cut off (ppb)	LOQ (ppb)
Cocoa natural powder	≤2	2
Cocoa alkalized sample (low and high fat)	≤2	2

13. LIABILITY

Samples evaluated as positive using the kit have to be re-tested with a confirmation method.

Gold Standard Diagnostics Budapest Kft 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.

Gold Standard Diagnostics Budapest Kft. shall not be liable for the unsafe use of the kit out of the current European safety regulations.

14. LITERATURE

Rosar G., Parisi E., Diana F. Performance comparison between classical and master-curve calibrated enzyme immunoassays. Poster presentation at RME 2021, 13th conference, 2021, February 1-3, virtual meeting.