B ZERO H2DON

Enzyme immunoassay for the detection of Deoxynivalenol (Cat. nr. HU0040018 / HU0040038)

BZERO H2DON is a kit prepared for a competitive immunoenzymatic assay for the quantitative analysis of deoxynivalenol (DON).
The kit contains the procedure and the materials sufficient for 96 determinations **(Cat.nr. HU0040018)** or 48 determinations **(Cat.nr. HU0040038)**.
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 (maize, wheat, durum wheat, barley, whole oat, dehulled oat, brown rice), corn gluten meal, swine feed, DDGS, soybean meal, malted barley, wheat bran and middlings, rye.

Sample preparation
Grinding, water extraction, filtration, dilution.

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

Detection limit
0.2 ppm

Specificity	
Compound	**Cross-reactivity (%)**
3-acetyl-DON	> 100
DON	100
3-glucosyl-DON	51 ± 9
15-acetyl-DON	12 ± 2
Nivalenol	< 0,5

1. TEST PRINCIPLE

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

2. PROVIDED REAGENTS

Premixing microtiter plate: non-coated wells, blank.
Microtiter plate: coated with anti- DON 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 ppm of DON**.**
ATTENTION: the standard zero is provided only. B/B_0 values of calibration curve (0.2 – 8 ppm) are reported in the kit certificate of analysis.
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. HU0040018 96 det.	Cat.nr. HU0040038 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)
Std.0	3 ml	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
- NaCl

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

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- 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 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 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

WARNING: The extracts can only be used within 4 hours. The diluted extract has to be used within 15 minutes. Do not store the extract and dilutions for longer time

6.1 Cereals (maize, wheat, durum wheat, barley, dehulled oat, brown rice), corn gluten meal, swine feed, DDGS, malted barley

1) Mix carefully the sample to be analyzed 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	Extraction solution
50 g	250 ml 10% NaCl in water*
5 g	25 ml 10% NaCl in water *

It is suggested to weigh 50 gr in order to have a better representative analysis of the sample and to speed up the filtration step

*** Preparation of extraction solution 10% NaCl in water:**
For 100 ml of solution: dissolve 10 gr of NaCl in approximately 70 ml of deionized or distilled water, then add deionized or distilled water to 100 ml.

4) Shake thoroughly for 3 minutes.
5) Filter the sample (Whatman 1 filter) and collect the filtrate. **ATTENTION:** It is not recommended to centrifuge the sample, because centrifugation can give rise to less accurate results.
6) Dilute the extract 4 times in deionized or distilled water (for example: 100 µl of extract +300 µl of water).
7) If the sample is dosed >8 ppm, dilute the extract 6 times more in deionized or distilled water (for example: 100 µl of diluted extract +500 µl of water), in order to obtain a dosage range 1.2-48 ppm.

6.2 Whole oat, soybean meal

1) Mix carefully the sample to be analyzed 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	Extraction solution
50 g	250 ml 20% NaCl in water*
5 g	25 ml 20% NaCl in water *

It is suggested to weigh 50 gr in order to have a better representative analysis of the sample and to speed up the filtration step.

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

4) Shake thoroughly for 3 minutes.

5) Filter the sample (Whatman 1 filter) and collect the filtrate. **ATTENTION:** It is not recommended to centrifuge the sample, because centrifugation can give rise to less accurate results.
6) Dilute the extract 4 times in deionized or distilled water (for example: 100 µl of extract +300 µl of water).
7) If the sample is dosed >8 ppm, dilute the extract 6 times more in deionized or distilled water (for example: 100 µl of diluted extract +500 µl of water), in order to obtain a dosage range 1.2-48 ppm.

6.3 Rye

1) Mix carefully the sample to be analyzed 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	Extraction solution
50 g	250 ml water
5 g	25 ml water

It is suggested to weigh 50 gr in order to have a better representative analysis of the sample and to speed up the filtration step.

4) Shake thoroughly for 3 minutes.
5) Filter the sample (Whatman 1 filter) and collect the filtrate. **ATTENTION:** it is not recommended to centrifuge the sample, because centrifugation can give rise to less accurate results.
6) Dilute the extract 8 times in deionized or distilled water (for example: 100 µl of extract +700 µl of water).
7) The resulting dosage range is 0.4-16 ppm, thus the result obtained by interpolation on the calibration curve must be multiplied by a factor 2.

6.4 Wheat bran and middlings

1) Mix carefully the sample to be analyzed 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	Extraction solution
50 g	500ml 20% NaCl in water*
5 g	50 ml 20% NaCl in water *

It is suggested to weigh 50 gr in order to have a better representative analysis of the sample and to speed up the filtration step.

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

4) Shake thoroughly for 3 minutes.
5) Filter the sample (Whatman 1 filter) and collect the filtrate. **ATTENTION**: it is not recommended to centrifuge the sample, because centrifugation can give rise to less accurate results.
6) Dilute the extract 4 times in deionized or distilled water (for example: 100 µl of extract +300 µl of water).
7) The resulting dosage range is 0.4-16 ppm, thus the result obtained by interpolation on the calibration curve must be multiplied by a factor 2.

7. WORKING SOLUTIONS PREPARATION

0 Std: ready to 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 standard 0 and each 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.
   - 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-DON 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.

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- 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 60 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/B_0 values provided for each standard (0.2; 0.5; 2; 8 ppm) in the kit certificate of analysis in a semi-logarithmic system of coordinates against the DON 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 (ppm) already considers the sample dilution factor. If the sample was 6 times further diluted in order to obtain a dosage range 1.2-48 ppm, multiply the result read on the calibration curve by a factor 6.
- For rye, wheat bran and middlings the extraction procedure requires that the results have to be multiplied by 2 folds.

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.

10. 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 11).
If the values are outside the specifications given, then the results of the test are not assured, therefore DON 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.

11. KIT SPECIFICATIONS

11.1 Assay specification

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

11.2 Assay performance

Matrix	Cut off ppm	LOQ ppm
Maize	0.25	0.3
Wheat	0.25	0.3
Durum wheat	0.25	0.3
Barley	0.4	0.5
Whole oat	<0.2	0.5

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

12. 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.

13. LITERATURE

Rosar G., Diana F. Master curve calibrated assays for cost-effective, reliable and consistent analysis of mycotoxins: a "B ZERO" review. Poster presentation at WMF meets ASIA, the12th Conference of The World Mycotoxin Forum, 2020, January 13-15, Bangkok, Thailand.