B ZERO® DON v2

Enzyme immunoassay for the detection of deoxynivalenol (Cat.nr. HU0040008 / HU0040028)

B ZERO **DONv2** is a kit prepared for an immunoenzymatic assay for the quantitative analysis of deoxynivalenol (DON).
The kit contains the procedure and the materials sufficient for 96 determinations ***(Cat.nr. HU0040008)*** or 48 determinations ***(Cat.nr. HU0040028)*** zero standard included.
A microtiter plate or strip photometer (manual or automatic ELISA reader) is required for sample evaluation.

Analysable samples
Cereals, feed, DDGS, wheat bran and middlings.

Sample preparation
- Cereals (maize, wheat barley), feed, DDGS: grinding, extraction in methanol-water, filtration, dilution (optional).
- Durum wheat: grinding, extraction in water, centrifugation, dilution.
- Wheat bran and middlings: extraction in water, activated charcoal clean-up, filtration, dilution.

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

Detection limit
Maize, wheat, barley, feed, DDGS: 0.04 ppm
Durum wheat: 0.12 ppm
Wheat bran/middlings: 0.24 ppm

Specificity	
Compound	**Cross-reactivity %**
3-acetyl-DON	>100
DON	100
3-glucosyl-DON	64±16
15-acetyl-DON	2
Nivalenol	<4

1. TEST PRINCIPLE

The assay is performed in plastic microplate, coated with specific anti-DON antibodies. In the premixing wells the enzyme labelled deoxynivalenol and the zero standard solution or samples are mixed and then transferred into the anti-DON microtiter plate. During the first incubation, free deoxynivalenol in the sample and enzyme-labelled deoxynivalenol 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 deoxynivalenol concentration in the 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 vial containing 0 ppm of deoxynivalenol. **ATTENTION:** Only the standard zero is provided. B/B_0 values of calibration curve (0.04-5 ppm) are reported in the kit certificate of analysis.
Enzyme conjugate: 1 plastic bottle.
Washing-buffer 10x: 1 plastic bottle containing 50 ml.
Developing solution: 1 plastic bottle.
Stop solution: 1 glass bottle. White cap.

Component	Cat.nr. HU0040008 96 det.	Cat.nr. HU0040028 48 det.
Microtiter plate	96 wells (12 strips x 8 wells)	48 wells (6 strips x 8 wells)
Premixing 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

For sample preparation
- Balance
- Methanol (100% methanol for Durum wheat, wheat bran and middlings; 70% methanol for wheat, maize, barley, feed DDGS).
- Activated Charcoal (Honeywell Fluka C3345).
- Distilled or deionized water.
- NaCl (wheat, maize, feed).
- Mill (grinding).
- Shaker (optional).
- Centrifuge (durum wheat) or filter paper (Whatman 1) (wheat, durum wheat, maize, barley,,feed, DDGS). Glass fiber filters (Whatman 934AH) (wheat bran and middlings).

For assay implementation
- 20-200 µl micropipettes with suitable tips
- 50-300 µl multichannel micropipette with suitable tips
- Absorbent paper
- Microtiter plate or strip reader equipped with a 450 nm filter.

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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) No 1272/2008. *Please refer to S*afety Data Sheet *available on Eurofins Tecna and Eurofins Technologies website.*
- Handle the reagents with caution, avoiding contact with skin, eyes and mucous membranes.
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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

WARNING: 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 better representative analysis of the sample.

6.1 Wheat, maize and feed

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*

***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. **ATTENTION**: It is suggested to shake manually or by a mild extraction system: it is possible to use magnetic stirrer, vortex or blender, but in some cases they can cause overestimation.
5) Filter the sample and collect the filtrate.
6) If the samples is dosed >5 ppm, dilute the extract 5 times (1+4) in methanol 70%, in order to obtain a dosage range 0.2-25 ppm.

6.2 Durum wheat

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 250 ml of distilled water. **Alternatively:** weigh 5 g of ground sample and add 25 ml of distilled water.
4) Shake thoroughly for 15 minutes. **ATTENTION**: It is suggested to shake manually or by a mild extraction system: it is possible to use magnetic stirrer, vortex or blender, but in some cases they can cause overestimation.
5) Filter the sample and collect the filtrate. **ATTENTION**: The filtration step could be very slow and it is suggested to let the sample settle down before filtration. As alternative, we suggest to centrifuge the sample at 3500g for 5 minutes and recover the supernatant.
6) Dilute the extract 1:3 (1+2) with methanol 100% (ex. 100µl of supernatant + 200 µl of methanol 100%).
7) The resulting dosage range is 0.12-15 ppm, thus the result obtained by interpolation on the calibration curve must be multiplied by a factor 3.
8) If the samples are dosed >15 ppm, dilute the extract 5 times (1+4) in methanol 70%, in order to obtain a dosage range of 0.6-75 ppm.

6.3 DDGS

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 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.
4) Blend or shake thoroughly for 15 minutes. **ATTENTION**: It is suggested to shake manually or by a mild extraction system: it is possible to use magnetic stirrer, vortex or blender, but in some cases they can cause overestimation.
5) Filter the sample (Whatman 1) and collect the filtrate.
6) If the samples are dosed >5 ppm, dilute the extract 5 times (1+4) in methanol 70%, in order to obtain a dosage range of 0.2-25 ppm.

6.4 Wheat bran and middlings

1) Mix carefully the sample to be analysed in order to make it homogeneous.
2) Weigh 10 g of ground sample and add 100 ml of distilled water.
3) Shake thoroughly for 3 minutes. **ATTENTION**: It is suggested to shake manually or by a mild extraction system: it is possible to use magnetic stirrer, vortex or blender, but in some cases they can cause overestimation.
4) Let the sample settle down.

5) Transfer the supernatant in a vial containing the activated Charcoal (Sigma C3345), 2 mg for each ml of sample extract. For example: weight 10 mg of activated Charcoal and add 5 ml of supernatant.
6) Shake thoroughly for 30 seconds.
7) Immediately filter the sample on glass fiber filters (Whatman 934AH) and collect the filtrate.
8) Dilute the filtrate 1:3 (1+2) with methanol 100% (ex. 100µl of supernatant + 200 µl of methanol 100%).
9) The resulting dosage range is 0.24-30 ppm, thus the result obtained by interpolation on the calibration curve must be multiplied by a factor 6.

6.5 Barley

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 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.
4) Blend or shake thoroughly for 10 minutes. **ATTENTION**: It is suggested to shake manually or by a mild extraction system: it is possible to use magnetic stirrer, vortex or blender, but in some cases they can cause overestimation.
5) Filter the sample (Whatman 1) and collect the filtrate.
6) If the samples is dosed >5 ppm, dilute the extract 5 times (1+4) in methanol 70%, in order to obtain a dosage range of 0.2-25 ppm.

7. WORKING SOLUTIONS PREPARATION

Std 0: 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 wash thoroughly with tap water.

8. ASSAY PROCEDURE

1) Predispose the assay layout, recording standard 0 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 the standard 0 and each 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-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.
   - 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 Maximum Binding (B_0) is thus made equal to 100% and the absorbance values are quoted as percentage:

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

- Enter the B/B0 provided for each standard (0.04; 0.25; 1.25; 5 ppm) in the kit lot analysis certificate 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. For durum wheat the extraction procedure requires that the results have to be multiplied by 3 folds. For bran and middlings the results have to be multiplied by 6 folds
- If extract have been further diluted in order to obtain a larger dosage range, further multiply the results by a factor 5.

Please note: For the calibration, use the "point to point" curve; Excel spreadsheets are available on the Eurofins Tecna website and can be downloaded directly from the bottom of the product page.

10. RESULTS OF EVALUATION

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 the kit specifications (chapter 11).
If the values are out of specifications, then the results of the test are not assured, therefore the deoxynivalenol 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 do not emerge, contact our technical assistance.

WARNING: If any kind of substitution is requested, please STORE THE KIT at the temperature defined by this instruction. Replacements are only possible if the original component is returned.

11. KIT SPECIFICATIONS

11.1 Assay specification

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

11.2 Assay performance

LOQ	Wheat, maize, feed: 0.125 ppm Durum wheat barley: 0.25 ppm

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., Persic L., Bassani V., Gon F., Ranieri C., Diana F.. Effects of "master curve calibration" on the performances of some ELISAs for food contaminants. Poster presentation at 6th International Symposium on Recent Advances in Food Analysis, 2013, November 5-8, Prague, Czech Republic.

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