13. ORDERING INFORMATION

For ordering the deoxynivalenol (DON) ELISA kit please use code 5121DON.

14. LAST MUTATIONS

Chapter 3: LOD table.
Chapter 8.1 Dilution factor 5 is descpribed for several matrices.
Chapter 11: Calculation 8.1 adapted.

EuroProxima B.V.
Beijerinckweg 18
NL 6827 BN Arnhem
The Netherlands

TEL: + 31 26 3630364
FAX: + 31 26 3645111
Web-site:http://www.europroxima.com
E-mail: info@europroxima.com

DEOXYNIVALENOL ELISA

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A competitive enzyme immunoassay
for screening and quantitative analysis of
deoxynivalenol (DON) in various matrices

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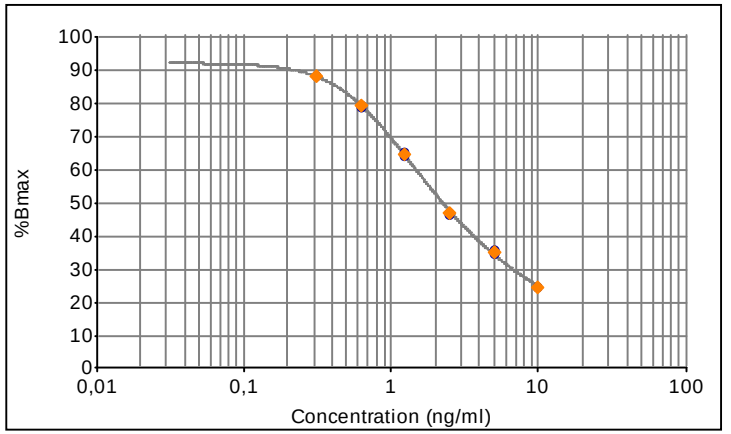


Figure 1 : Example of a DON calibration curve

The DON concentration in the extracts (ng/ml) corresponding to the % maximal absorbance of each extract can be read from the calibration curve.

8.1 Cereals, food, feed and silage samples

The DON-equivalents, as read from the calibration curve, have to be multiplied by the dilution factor 5 or 100, depending on which method is used.

8.2 Beer samples

The DON-equivalents, as read from the calibration curve, have to be multiplied by a factor 5 to obtain the DON content in beer samples.

12. LITERATURE

1. Moss M.O. : Mycotoxins. Mycol. Res. 1996, **100**, 513-523.

2. Miller J.D.: Fungi and mycotoxins in grain: implications for stored product research. J. Stored Prod. Res. 1995, **31**, 1-16.

3. Tanaka T., Hasegawa A., Yamamoto S., Lee U., Sugiura Y. and Ueno Y.: Worldwide contamination of cereals by the Fusarium mycotoxins nivalenol, deoxynivalenol and zearalenone. 1. Survey of 19 countries. J. Agric. Food Chem. 1988, **36**, 979-983.

3. Pipette 50 µl of each sample solution in duplicate into the remaining wells of the microtiter plate.

4. Pipette 25 µl of conjugate (DON-HRP) into all wells, except wells H1 and H2.

5. Pipette 25 µl of antibody solution into all wells, except wells H1 and H2.

6. Seal the microtiter plate and shake the plate for 1 minute on a microtiter plate shaker.

7. Incubate the plate for 1 hour in the dark at 4°C (2°C to 8°C).

8. Discard the solution from the microtiter plate and wash 3 times with rinsing buffer.

9. Pipette 100 µl of substrate solution into each wells.

10. Incubate for 30 minutes in the dark at 20°C to 25°C.

11. Pipette 100 µl of stop solution into each well.

12. Read the absorbance values immediately at 450 nm.

11. INTERPRETATION OF RESULTS

Subtract the mean optical density (O.D.) of the wells H1 and H2 (Blank) from the individual O.D. of the wells containing the standards and the samples.

The O.D. values of the six standards and the samples (mean values of the duplicates) are divided by the mean O.D. value of the zero standard (Bmax, wells A1 and A2) and multiplied by 100. The zero standard (Bmax) is thus made equal to 100% (maximal absorbance) and the other O.D. values are quoted in percentages of the maximal absorbance.

$$\frac{\text{O.D. standard (or sample)}}{\text{O.D. zero standard (Bmax)}} \times 100\% = \%\ \text{of maximal absorbance}$$

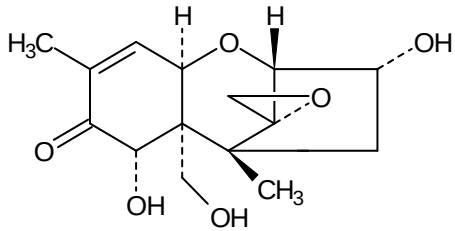
Calibration curve:
The values (% maximal absorbance) calculated for the standards are plotted on the Y-axis versus the analyte equivalent concentration (ng/ml) on a logarithmic X-axis.

Alternative for calibration curve:
The absorption value of the standards is plotted on the Y-axis versus the concentration on the X-axis. The Y-axis is in logit the Y-axis is logarithmic.

BRIEF INFORMATION

The deoxynivalenol (DON) ELISA is a competitive enzyme immunoassay for the screening of food, feed and other biological samples. The test is based on antibodies directed against deoxynivalenol-HSA. The antiserum is highly specific for the mycotoxin DON and its metabolite 3-acetyl-DON. The ELISA kit contains a 96 well microtiter plate as well as all essential reagents including ready-to-use standards to perform the test. Methods for a fast and efficient extraction of DON from different matrices are included in the kit manual.

1. INTRODUCTION



Chemical structure of deoxynivalenol

Deoxynivalenol belongs to the group of trichothecenes produced essentially by a broad range of Fusarium moulds of which *F. graminearum* and *F. culmorum* are the most important [1]. These fungi typically develop during prolonged cool, wet growing and harvest seasons to produce *Fusarium* head blight in cereal crop. Deoxynivalenol, also known as 'vomitoxin', because of its potent emetic effects in swine, is most often associated with *Fusarium* head blight. Some fungal strains make DON by a 3-acetylated precursor (mainly in Europe and Asia) and others produce a 15-acetylated precursor (mainly in North and South America) [2]. The amount of 3-acetyl-DON and/or 15-acetyl-DON in a sample is approximately 10% of the amount of DON present.

Significant concentrations of DON are frequently detected in wheat, barley, corn and oats, while lower levels are usually found in rye, sorghum and rice. About 40-50% of all samples analysed were found positive for DON with average contents of 290 µg/kg (range 2 - 10.000 µg/kg) [3].

This assay is extremely suitable for screening purposes on the presence of the mycotoxin DON in food and feed products, but also in liquid samples as beer.
For confirmation purposes a previous HPLC isolation in combination with mass-spectrometry analysis is necessary.

2. PRINCIPLE OF THE DON-ELISA

The kit is based on a microtiter plate (12 strips, each 8 wells), precoated with sheep antibodies to rabbit IgG. A specific antibody (rabbit anti-DON-HSA), enzyme labelled DON (DON-HRP) and DON standard or sample are added to the pre-coated wells followed by a single incubation step. The specific antibodies are bound by the immobilized antibodies and at the same time free DON (present in the standard solution or in the sample) and enzyme labelled DON compete for the specific antibody binding sites (competitive enzyme immunoassay). After an incubation time of one hour, the non-bound (enzyme labelled) reagents are removed in a washing step. The amount of bound DON enzyme conjugate is visualized by the addition of a chromogen substrate (tetramethylbenzidine, TMB). Bound enzyme conjugate transforms the chromogen into a coloured product.
The substrate reaction is stopped by the addition of sulfuric acid. The colour intensity is measured photometrically at 450 nm and is inversely proportional to the DON concentration in the sample.

3. SPECIFICITY AND SENSITIVITY

The DON ELISA utilizes a specific antibody raised in rabbits against protein conjugated DON. The cross-reactivity pattern of this antibody is:

Cross-reactions:	Deoxynivalenol (DON)	100%
	3-Acetyl-deoxynivalenol	96%
	15-Acetyl-deoxynivalenol	< 0.1%
	Nivalenol	40%
	T-2 toxin	< 0.1%
	Zearalenone	< 0.1%
	Fumonisine	< 0.1%

Due to the cross-reaction of the antiserum with 3-acetyl-DON, that might be present in a positive DON sample for maximally 10 – 15% relative to the DON concentration, an overestimation can be expected of the DON concentration of maximally 10 – 15%.

The Limit of detection (LOD) is calculated as: Xn+3SD and is determined under optimal conditions.

Matrix	Procedure	LOD dilution factor 5	LOD dilution factor 100
Cereals	8.1	1.5 ppb	30 ppb
Food	8.1		30 ppb
Feed	8.1		30 ppb
Beer	8.2	1.5 ppb	
Silage	8.1		50 ppb

Rinsing buffer
The rinsing buffer is delivered 20x concentrated. Prepare dilutions freshly before use. Per strip 40 ml of diluted rinsing buffer is used (2 ml concentrated rinsing buffer + 38 ml distilled water).

Substrate solution
The substrate solution (ready-to-use) precipitates at 4°C. Take care that this vial is at 20°C to 25°C (keep in the dark) and mix the content before pipetting in the wells.

10. ASSAY PROCEDURE

Rinsing protocol

In ELISA's, between each immunological incubation step, unbound components have to be removed efficiently. This is reached by appropriate rinsing. It should be clear that each rinsing procedure must be carried out with care to guarantee good inter- and intra-assay results.
Basically, manual rinsing or rinsing with automatic plate wash equipment can be performed as follows:

Manual rinsing

1. Empty the contents of each well by turning the microtiter plate upside down and remove residual liquid by striking the plate against a paper towel.
2. Fill all the wells to the rims (300 µl) with rinsing solution.
3. This rinsing cycle (1 and 2) should be carried out 3 times.
4. Turn the plate upside down and empty the wells by a firm short vertical movement.
5. Place the inverted plate on absorbent paper towels and tap the plate firmly to remove residual rinsing solution from the wells.
6. Take care that none of the wells dry out before the next reagent is dispensed.

Rinsing with automatic microtiter plate wash equipment

When using automatic plate wash equipment, check that all wells can be aspirated completely, that the rinsing solution is nicely dispensed reaching the rim of each well during each rinsing cycle. The washer should be programmed to execute three rinsing cycles.

Assay Protocol

1. Prepare samples according to Chapter 8 (Sample preparation) and prepare reagents according to Chapter 9 (Preparation of reagents).

2. Pipette 100 µl of the zero standard in duplicate (wells H1, H2, blank).
   Pipette 50 µl of the zero standard in duplicate (wells A1, A2, Bmax).
   Pipette 50 µl of each of the DON standard solutions in duplicate (wells B1,2 to G1,2 i.e. 0.313, 0.625, 1.25, 2.5, 5 and 10 ng/ml).

8. SAMPLE PREPARATIONS

8.1 Cereals, food, feed and silage (100 x diluted)
- Grind and pulverize approximately 50 - 100 g of sample to obtain a homogeneous mixture or powder
- Weigh out an amount of 1 g of the grinded sample and add 19 ml of distilled water (1:20)
- Shake thoroughly (e.g. 5 minutes using a vortex)
- Filtrate a part of the water extract using a filter paper
- Dilute 50 µl of the filtrate with 200 µl of dilution buffer (final dilution 1:100) Pipette 50 µl of this dilution in the ELISA test.

Cereals, food, feed and silage (5 x diluted)
- Grind and pulverize approximately 50 - 100 g of sample to obtain a homogeneous mixture or powder
- Weigh out an amount of 1 g of the grinded sample and add 4 ml of distilled water.
- Shake thoroughly (e.g. 5 minutes using a vortex)
- Filtrate a part of the water extract using a filter paper
- Pipette 50 µl of this dilution in the ELISA test.

8.2 Beer
Beer is degassed in an ultrasonic bath. An aliquot of 50 µl of the degassed sample is diluted with 200 µl of dilution buffer. An aliquot of 50 µl is used in the ELISA test.

9. PREPARATION OF REAGENTS

Before starting the test, allow the reagents to come to ambient temperature. Any reagents not used should be put back into storage immediately at 2°C to 8°C.

Microtiter plate
Return unused strips into the resealable bag with desiccant and store at 2°C to 8°C for use in subsequent assays. Retain also the strip holder.

Conjugate solution
Reconstitute the vial of lyophilized conjugate (DON-HRP) with 4 ml dilution buffer, mix thoroughly and keep in the dark until use. Store the vial immediately after use in the dark at 2°C to 8°C.

Antibody solution
Reconstitute the vial of lyophilized antibodies with 4 ml dilution buffer, mix thoroughly and keep in the dark until use. Store the vial immediately after use in the dark at 2°C to 8°C.

Note: For prolonged storage of antibody and conjugate store aliquots at –20ºC

4. HANDLING AND STORAGE

- Kit and kit components are stored in a refrigerator (2°C to 8°C) before and immediately after use.
- After the expiry date of the kit and/or components has passed, no further quality guarantee is valid.
- Bring all kit components including the microtiter plate at ambient (room) temperature before use.
- Avoid condensation in the wells of the plate. Bring the sealed plate at ambient temperature before opening the plate sealing.
- Any direct action of light on the chromogen solution should be avoided.

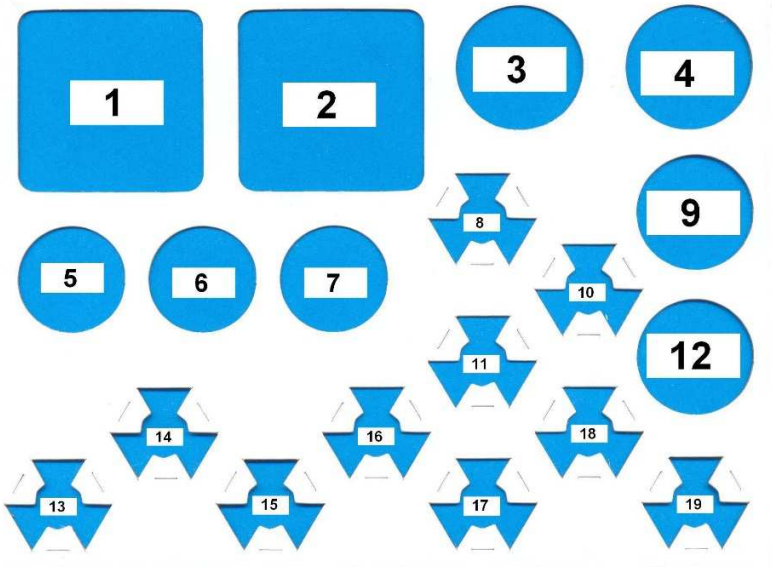
Degeneration of the reagents may have occurred when the following phenomena are observed:
- A blue colouring of the chromogen solution before transferring it into the wells.
- A weak or absent colour reaction of the maximum binding (zero standard) (E450nm < 0.8).

5. KIT CONTENTS

Manual

One sealed microtiter plate (12 strips, 8 wells each), coated with antibodies to rabbit IgG. Plate is ready-to-use.

Position of the reagents in the kit. For preparation of the reagents see Chapter 9.



1. **Dilution buffer** (40 ml, ready-to-use)
2. **Rinsing buffer** (30 ml, 20x concentrated)
3. **Substrate solution** (12 ml, ready-to-use)
4. **Stop solution** (15 ml, ready-to-use)
5. **Conjugate** (lyophilized, blue cap)
6. **Antibody** (lyophilized, yellow cap)
7. not in use
8. not in use
9. not in use
10. not in use
11. not in use
12. not in use
13. **Zero Standard** (2ml, ready-to-use)
14. **Standard solution 1** (1ml, ready-to-use) **0.313 ng/ml**
15. **Standard solution 2** (1ml, ready-to-use) **0.625 ng/ml**
16. **Standard solution 3** (1ml, ready-to-use) **1.25 ng/ml**
17. **Standard solution 4** (1ml, ready-to-use) **2.5 ng/ml**
18. **Standard solution 5** (1ml, ready-to-use) **5.0 ng/ml**
19. **Standard solution 6** (1ml, ready-to-use) **10 ng/ml**

6. EQUIPMENT AND MATERIALS REQUIRED BUT NOT PROVIDED

- Scales and weighing vessels
- Gloves
- Fume hood
- Homogeniser (vortex, mixer)
- Centrifuge (2000 x g)
- Automated microtiter plate washer or 8-channel micropipette 100 – 300 µl
- Microtiter plate shaker
- Microtiter plate reader with 450 nm filter
- Micropipettes, 100 – 1000 µl
- Multipipette with 2.5 ml combitips
- disposable tubes
- tubes with screw cap (Greiner, polypropylene)
- distilled water
- filter paper
- ultrasonic bath

7. PRECAUTIONS

- DON is a toxic compound. Avoid contact with mouth and skin.
- Any material contaminated with DON should be destroyed or decontaminated by addition of sodium hydochlorite solution (10% v/v).
- The stop reagent contains 0.5 M sulfuric acid. Do not allow the reagent to get into contact with the skin.
- Avoid contact of all biological materials with skin and mucous membranes.
- Do not pipette by mouth.
- Do not eat, drink, smoke, store or prepare foods, or apply cosmetics within the designated work area.
- TMB is toxic by inhalation, in contact with skin and if swallowed; observe care when handling the substrate.
- Do not use components past expiration date and do not intermix components from different serial lots.
- Each well is ultimately used as an optical cuvette. Therefore, do not touch the under surface of the wells, prevent damage and dirt.
- All components should be completely dissolved before use. Take special attention to the substrate, which crystallises at 4°C.
- Optimal results will be obtained by strict adherence to this protocol. Careful pipetting and washing throughout this procedure are necessary to maintain good precision and accuracy.