T-2/HT-2 TOXIN ELISA

5121THT[3]07.20

A competitive enzyme immunoassay
for quantitative analysis of T-2 and HT-2
toxins in food and feed samples

EUROPROXIMA T-2/HT-2TOXIN ELISA

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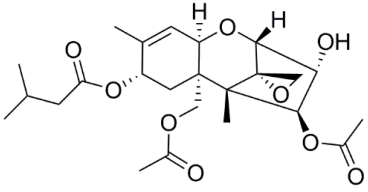
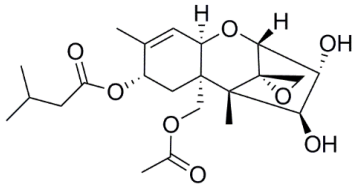
BRIEF INFORMATION

The T-2/HT-2 toxin ELISA is a competitive enzyme immunoassay for the quantitative detection of T-2 and HT-2 mycotoxins in cereals and baby food. The test is based on an antibody that has a high affinity for both T-2 and HT-2 toxins. Samples and standards are measured in duplicate, which means that in total 40 samples can be analysed with one kit. The ELISA kit contains all the reagents, including standards, required to perform the test. Materials and chemicals required for sample extraction are not included in the test kit.

1. INTRODUCTION



T-2 toxin



HT-2 toxin

T-2 toxin and its metabolite HT-2 toxin belong to the group of trichothecenes, a large group of mycotoxins produced mainly by *Fusarium* species. Both T-2 and HT-2 toxins can be found in crops such as corn, wheat, barley and oats and also in products thereof. These toxins are harmful to both humans and animals. Cats and pigs are among the most sensitive species. T-2 and HT-2 impair protein and DNA synthesis and interfere with both red and white blood cells formation in bone marrow which leads to a number of adverse effects. Due to these toxic effect the Panel on Contaminants in the Food Chain (CONTAM Panel) of the European Food Safety Authority has set the tolerable daily intake for the sum of both T-2 and HT-2 toxins at 100 ng/kg body weight.

In the EU, according to the Commission Recommendation 2013/165/EU [2], Member States with involvement of food and feed business operators are required to monitor for the presence of both T-2 and HT-2 toxins in food and feed. The indicative limits for T-2 and HT-2 toxins have been also established based on the occurrence data. These are 100–1000 µg/kg for unprocessed cereals, 50–200 µg/kg for cereal grains for direct human consumption, 25–200 µg/kg for cereal products for human consumption, 15 µg/kg for infant food and 250–2000 µg/kg for feed. If the sample is found to be contaminated above these values it is required to perform an investigation in order to identify factors causing these high levels. A limit of detection of an analytical screening method for the sum of T-2 and HT-2 toxins should preferably not be higher than 25 µg/kg.

2. PRINCIPLE OF THE T-2 TOXIN ELISA

The microtiter plate based ELISA kit consists of 12 strips, each containing 8 wells, precoated with rabbit antibodies to mouse IgG. Specific antibodies (mouse monoclonal anti-T-2 toxin), horseradish peroxidase labelled T-2 toxin (enzyme conjugate T-2 toxin-HRP) as well as T-2 toxin standard solutions or samples are added to the precoated wells. The specific antibodies are captured by the immobilised rabbit anti-mouse antibodies. At the same time the T-2 toxin-HRP and the T-2/HT-2 toxin present in the standard solutions or in the samples compete for the specific anti-T-2 toxin antibody binding sites. After one hour incubation, the non-bound (enzyme labelled) reagents are removed by washing. The amount of bound T-2 toxin-HRP is visualised by the addition of enzyme substrate/chromogen (peroxide/tetramethylbenzidine, TMB). During the incubation the colourless chromogen is converted by the enzyme into a blue reaction product. The intensity of this blue colour is inversely proportional to the amount of bound T-2/HT-2 toxin. The more T-2/HT-2 toxin is present in the standard solution or sample, the less colour is developed. The enzymatic reaction is stopped by the addition of sulphuric acid. In the acidic environment the blue colour changes into a yellow colour. The colour intensity is measured photometrically at 450 nm.

3. SPECIFICITY AND SENSITIVITY

The T-2/HT-2 toxin ELISA utilizes monoclonal antibodies raised in mouse to protein conjugated T-2 toxin. The cross-reactivity pattern of the antibody is:

T-2 toxin	100%
HT-2 toxin	125%

The cross-reactivities are determined in a buffer system. The reported values may be different in samples due to matrix effects.
The test cannot discriminate between analytes and cross-reactive substances.

The Limit of detection (LOD) is determined under optimal conditions. Cut-off criteria need critical consideration.

Matrix	LOD [ppb]
Cereals	12.0
Baby food	6.0

If the sample is found to be non-compliant, the results shall be verified by re-analysis of the sample using a confirmatory method.

4. HANDLING AND STORAGE

- Kit and kit components should be stored at 2°C to 8°C in a dark place. For repeated use store kit components as specified under chapter 9.
- 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 to ambient (room) temperature before use.
- Dilute the kit components immediately before use, but after the components are brought to ambient temperature.
- Avoid condensation in the wells of the plate. Bring the sealed plate to ambient temperature before opening the plate sealing.
- The substrate chromogen solution can be stored in a refrigerator (2ºC to 8ºC) until the expiry date stated on the label.
- Exposure of the chromogen solution to light should be avoided.

Degeneration of the reagents may have occurred when the following phenomena are observed:

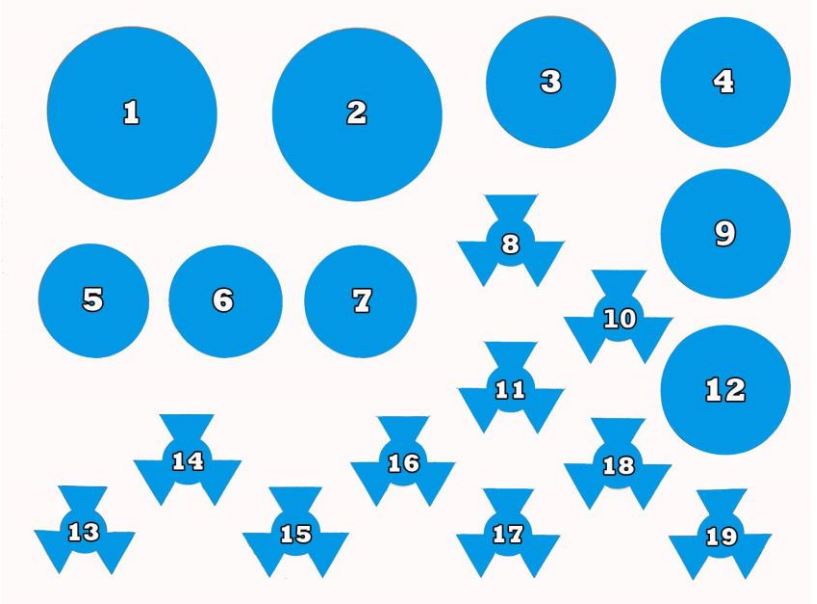
- A blue colouring of the chromogen solution before transferring it into the wells.
- A weak or no colour reaction in the zero standard wells (E450nm < 0.8).

4. KIT CONTENTS

Manual

1 sealed microtiter plate (12 strips, 8 wells each) coated with antibodies to mouse IgG, ready-to-use.

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



1. **Dilution buffer** (20 ml, 4x concentrated)
2. **Rinsing buffer** (30 ml, 20x concentrated)
3. **Substrate solution** (12 ml, ready-to-use)
4. **Stop solution** (15 ml, ready-to-use)
5. not in use
6. not in use
7. not in use
8. **Conjugate** (100x concentrated, blue cap)
9. not in use
10. **Antibody** (100x concentrated, yellow cap)
11. not in use
12. not in use
13. **Zero standard** (2ml, ready-to-use)
14. **Standard solution 1** (1ml, ready-to-use) **0.125 ng/ml**
15. **Standard solution 2** (1ml, ready-to-use) **0.25 ng/ml**
16. **Standard solution 3** (1ml, ready-to-use) **0.5 ng/ml**
17. **Standard solution 4** (1ml, ready-to-use) **1.0 ng/ml**
18. **Standard solution 5** (1ml, ready-to-use) **2.0 ng/ml**
19. **Standard solution 6** (1ml, ready-to-use) **4.0 ng/ml**

6. EQUIPMENT AND MATERIALS REQUIRED BUT NOT PROVIDED

- Scales and weighing vessels
- Gloves
- Fume hood
- Homogeniser
- Vortex mixer
- Centrifuge (4300 x g)
- Automated microplate washer or 8-channel micropipette 100 – 300 µl
- Microtiter plate shaker
- Microtiter plate reader with 450 nm filter
- Micropipettes 20 – 200 µl, 100 – 1000 µl
- Multipipette with 2.5 ml combitips
- Aluminium foil or parafilm
- Methanol
- Deionized or distilled water

7. PRECAUTIONS

- This kit may contain hazardous substances. For hazard notes please refer to the appropriate safety data sheets (SDS).
- 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.
- Do not use components past expiration date and do not use components from different 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 and rinsing buffer, which crystallize at +4ºC.
- 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.

8. SAMPLE PREPARATION

Cereals and baby food

- Grind or pulverise 50–100 g of a sample into a fine powder or homogeneous mixture.
- Weigh out 1 g of the homogenized sample in a 15 ml tube with a screw cap.
- Add 5 ml of methanol/water (40:60, v/v).
- Vortex the mixture for 1 minute.
- Centrifuge the tubes with mixture at 4300xg for 10 minutes.
- Dilute the clear supernatant:
  for cereals: mix 25 µl of the supernatant with 475 µl of the dilution buffer (final dilution factor 120x)
  for infant food: mix 50 µl of the supernatant with 450 µl of the dilution buffer (final dilution factor 60x).
- Use 50 µl of this solution in the ELISA test.

9. PREPARATION OF REAGENTS

Before beginning the test, allow the reagents to come to ambient temperature. Any reagents not used should be put back into storage immediately at +2ºC to +8ºC.
Prepare reagents freshly before use

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

Dilution buffer
The dilution buffer is 4 times concentrated. The concentrated buffer should be at room temperature and thoroughly mixed before diluting (20 ml buffer + 60 ml distilled water). The concentrated buffer can show precipitates of the content. Mix well before dilution with distilled water.

Conjugate solution
The conjugate is 100x concentrated. Spin down the conjugate in the vial by a short centrifugation step (1 min., 1000 x g). Add 5 µl of the concentrated conjugate solution to 495 µl of the dilution buffer. Per 2 x 8 wells 400 µl is required. Store unused concentrated conjugate at +2°C to +8°C.

Antibody
The antibody is 100x concentrated. Spin down the antibody in the vial by a short centrifugation step (1 min. 1000 x g). Add 5 µl of the concentrated antibody to 495 µl of the dilution buffer. Per 2 x 8 wells 400 µl of antibody solution is required. Store concentrated antibody immediately upon use at +2°C to +8°C.

Rinsing buffer
The rinsing buffer is 20 times concentrated. Prepare fresh dilution before use. For each strip 20 ml of diluted rinsing buffer is used (1 ml concentrated rinsing buffer + 19 ml distilled water).

Substrate solution
The substrate solution (ready-to-use) precipitates at 4°C.
Bring the vial at room temperature (20°C to 25°C, kept in the dark) and mix it well before pipetting into the wells.

10. ASSAY PROCEDURE

Rinsing protocol

Unbound components have to be removed efficiently between each incubation step in ELISAs. This is achieved by appropriate rinsing. Each rinsing procedure must be carried out with care to guarantee good inter- and intra-assay results.
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. Do not allow the wells dry out before the next reagent is dispensed.

Rinsing with automatic microtiter plate wash equipment

When using automatic plate wash equipment, make sure that all wells can be aspirated completely and 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 and prepare reagents according to chapter 9.

2. Pipette 100 µl of dilution buffer in duplicate (well H1, H2).
   Pipette 50 µl of dilution buffer in duplicate (well A1, A2).
   Pipette 50 µl of each standard dilution in duplicate (well B1,2 to G1,2 i.e. 0.125, 0.25, 0.5, 1.0, 2.0 and 4.0 ng/ml).

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

4. Add 25 µl of conjugate (T-2 toxin-HRP) to all wells, except wells H1 and H2.

5. Add 25 µl of antibody to all wells, except wells H1 and H2.

6. Seal the microtiter plate and shake the plate for a few seconds.

7. Incubate for 1 hour in the dark at 37°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 well. Incubate 30 minutes at room temperature (20ºC - 25ºC) in the dark.

10. Add 100 µl of stop solution to each well.

11. 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.

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

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 O.D. values of the standards are plotted on the Y-axis versus the concentration on the X-axis. The scale of the Y-axis is logit and the X-axis is logarithmic.

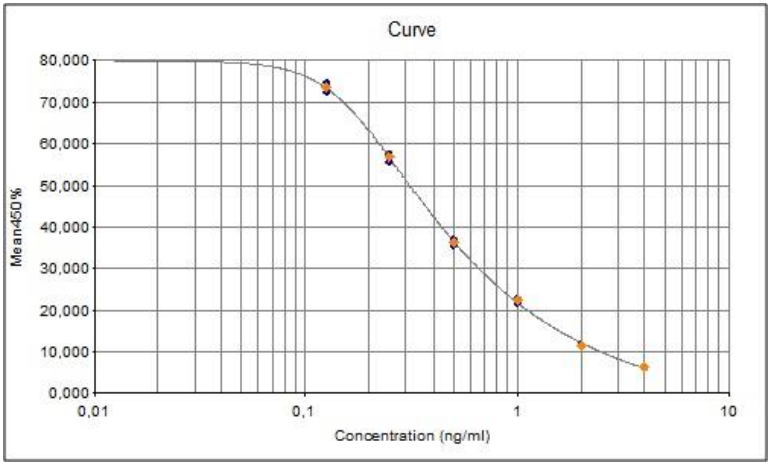


Figure 1 : Example of a calibration curve

The amount of T-2/HT-2 toxin in the samples is expressed as T-2 toxin equivalents. The T-2 toxins equivalents in the extracted samples (ng/ml) corresponding to the percentage maximal absorbance of each extract can be read from the calibration curve.

Baby food

The T-2 toxin equivalents read from the calibration curve have to be multiplied by a factor 60 to obtain the T-2/HT-2 toxin content in baby food samples expressed in ppb (ng/g).

Cereals

The T-2 toxin equivalents read from the calibration curve have to be multiplied by a factor 120 to obtain the T-2/HT-2 toxin content in cereals expressed in ppb (ng/g).

12. LITERATURE

European Food Safety Authority (EFSA). Scientific Opinion on the risks for animal and public health related to the presence of T-2 and HT-2 toxin in food and feed. (2011) EFSA Journal 9(12):2481.

Commission Recommendation 2013/165/EU on the presence of T-2 and HT-2 toxin in cereals and cereal products. (2013) Official Journal of the European Union L91/12.

13. ORDERING INFORMATION

For ordering the T-2/HT-2 toxin ELISA kit, please use cat. code 5121THT.

14. REVISION HISTORY

The manual is adapted to a new layout of the test kit. Several textual changes are added.

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