TETRODOTOXIN (TTX) ELISA

5191TTX[3]12.18

A competitive enzyme immunoassay
for the quantitative analysis of tetrodotoxin in
fish and shellfish

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BRIEF INFORMATION

The Tetrodotoxin (TTX) ELISA is a competitive enzyme immunoassay for the quantitative detection of TTX in fish and shellfish. With this ELISA-kit 96 analyses can be performed. Samples and standards are measured in duplicate which means that a total of 40 samples can be analysed.
The TTX ELISA contains all reagents to perform the test. Reagents for sample preparation are not included in the kit.

1. INTRODUCTION

Tetrodotoxin (TTX) and its analogues belong to a group of neurotoxins that are produced by various marine bacteria. The toxin can accumulate in certain species of fish, different marine bivalves (clams, oysters and mussels) and gastropods. Due to the worldwide increase in water temperature TTX has appeared also in the European waters. TTX has been recently detected in seafood harvested in the United Kingdom, Portugal, Spain, Greece and the Netherlands. The ingestion of contaminated seafood can have fatal consequences. On the cellular level TTX causes blockage of voltage-gated sodium channels that leads to alteration of neuronal functions and muscle paralysis. Death can occur due to heart or respiratory failure. The majority of the poisoning cases have been caused by the consumption of pufferfish contaminated with TTX in Japan, where a limit of 2000 µg/kg was set for fish. As for now there are no maximum limits for TTX in the European Union. According to the recent European Food Safety Authority Scientific Opinion the concentration of TTX and its analogues of 44 µg/kg of shellfish meat should not result in adverse effects in humans.

2. PRINCIPLE OF THE TETRODOTOXIN (TTX) ELISA

The microtiter plate based TTX ELISA consists of one plate (12 strips, 8 wells each) pre-coated with TTX.
A mouse monoclonal antibody, a standard and samples are added to the pre-coated wells.
Simultaneously TTX present in the standard solutions or in the samples and immobilized TTX on the surface of the wells compete for binding to the antibody (competitive enzyme immunoassay). After incubation, non-bound reagents are removed in a washing step.
The bound antibody TTX complex is detected by addition of HRPO labeled anti mouse antibody. After a washing step, the amount of bound complex is visualized by addition of enzyme/chromogen (peroxide/tetramethylbenzidine, TMB). During the incubation the colourless chromogen is converted by the enzyme into a blue reaction product.
The colour intensity is inversely proportional to the amount of bound TTX, i.e. the more TTX present in the standard solution or sample, the less colour is developed.
The colour development is stopped by addition of sulfuric acid. By which the blue

colour is converted into a yellow colour which intensity is measured photometrically at 450 nm.

3. SPECIFICITY AND SENSITIVITY

For this TTX ELISA a specific antibody is used, obtained by immunisation of mice with a TTX protein conjugate. The cross-reactivity panel of the antibody (as tested in buffer) is:

TTX	100%
Okadaic acid	0%
Saxitoxin	0%
Domoic acid	0%

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) and the detection capability (CCβ) are determined under optimal conditions. Cut off criteria need critical consideration

Matrix	**Procedure**	LOD (ng/g)	CCβ (ng/g)
Fish	8.1	6.7	20
Shellfish	8.2	9.4	20

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

- Store the kit at +2°C to +8°C in a dark place.
- After the expiry date (see kit label) has passed, the kit performance is no longer guaranteed.
- Before opening the sealed plate, the plate should be at ambient temperature, to avoid condensation in the wells. Return the unused strips into the zip resealable bag with desiccant and store at +2°C to +8°C
- The substrate, the standard solutions and the 100 times concentrated enzyme conjugate (HRP) can be stored in a refrigerator (+2°C to +8°C) until the expiry date stated on the label.
- 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:

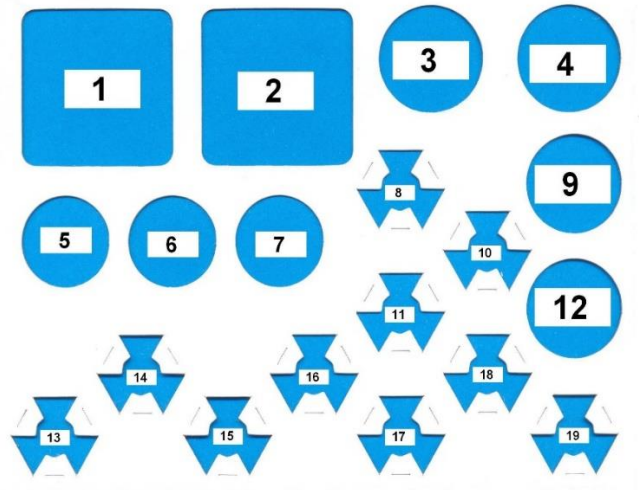
- A blue colouring of the chromogen solution before transferring it into the wells.
- A weak or absent colour reaction of the zero standard (maximum binding) (E450nm < 0.8).

5. KIT CONTENTS

Manual

One sealed (96-wells) microtiter plate (12 strips, 8 wells each), coated with gliadin. Ready-to-use.

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



1. **Standard/Sample 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** (12 ml, ready-to-use)
5. **Standard** (lyophilized)
6. **Standard** (lyophilized)
7. **Standard** (lyophilized)
8. **Conjugate (150 µl, 100x concentrated)**
9. **Dilution buffer** (13 ml, ready-to-use)
10. **TTX antibody (100 µl, 100x concentrated)**
11. Not in use
12. **Dilution buffer** (13 ml, ready-to-use)
13. Not in use
14. Not in use
15. Not in use
16. Not in use
17. Not in use
18 Not in use
19 Not in use

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

8.1 Fish
- Mix 1 gram of the homogenized sample with 5 ml NaAcetate buffer* (pH4.8)
- Vortex shortly and add 3 ml Ethylacetate
- Vortex shortly and then mix head over head for 5 minutes
- Centrifuge 5 minutes at 4000 x g and 20-25°C
- Transfer 2 ml of the underlayer carefully to a new tube.
- Dilute this 4 times with standard/sample dilution buffer: mix 50 µl with 150 µl buffer.
- Mix well and use 50µl directly in the ELISA

8.2 Shellfish
- Mix 1 gram of the homogenized sample with 5 ml NaAcetate buffer* (pH4.8)
- Vortex shortly and then mix head over head for 5 minutes
- Centrifuge 5 minutes at 4000 x g and 20-25°C
- Dilute the upper layer 4 times with standard/sample dilution buffer: mix 50 µl with 150 µl buffer.
- Mix well and use 50µl directly in the ELISA

9. PREPARATION OF REAGENTS

Before beginning the test, the reagents should be brought up to room 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 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.

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

Dilution buffer
The dilution buffer for the conjugate and antibody is supplied ready-to-use in the kit (2 vials)

Standard/Sample dilution buffer
The standard/sample buffer for the dilution of the standards and samples is supplied ready-to-use in the kit.

Conjugate solution (150 µl)
The conjugate is delivered 100x concentrated. Spin down the conjugate in the vial by a short centrifugation step (1 min., 1000 x g). Add 20 µl of the concentrated conjugate solution to 1980 µl of dilution buffer. Per 2 x 8 wells 1.6 ml of diluted conjugate is required. Store unused concentrated conjugate at +2ºC to +8ºC.

Antibody (100 µl)
The antibody is delivered 100X concentrated. Spin down the antibody in the vial by a short centrifugation step (1 minute, 1000 x g). Add 10 µl of the concentrated conjugate solution to 990 µl of dilution buffer. Per 2 x 8 wells 800 µl of diluted antibody is required. Store unused concentrated conjugate at +2ºC to +8ºC.

Substrate/chromogen solution
The substrate/chromogen solution (ready-to-use) tends to precipitate at +4ºC. Take care that this vial is at room temperature when used (keep in the dark) and mix the content before pipetting into the wells.

Standards
Prepare a dilution range of TTX standards. Add 2 ml of standard/sample dilution buffer to the TTX
standard and mix. This solution contains 20 ng TTX per ml. Pipette 0.25 ml of this solution into a clean tube and add 0.25 ml of standard/sample dilution buffer. Continue to make a dilution range of 20, 10, 5, 2.5, 1.25 and 0.625 ng/ml.

For prolonged storage: freeze aliquots at -20ºC.

*NaAcetate buffer
Mix 300 ml of 0.1M sodiumacetate solution with 200 ml of 0.1M acetic acid solution. Check and, in necessary, set the pH at 4.8.

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 followed by a firm short vertical movement.
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 washing solution in 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, maximal signal).
   Pipette 50 µl of each of the standard solutions in duplicate (wells B1,2 to G1,2 i.e. 20, 10, 5, 2.5, 1.25, 0.625 ng/ml)

3. Pipette 50 µl of each sample solution in duplicate into the remaining wells of the microtiter plate (40 samples; 80 wells).

4. Pipette 50 µl of antibody into all wells, except the blank H1 and H2.

5. Seal the microtiter plate and shake the plate for a few seconds on a microtiter plate shaker.

6. Incubate for 30 minutes in the dark at room temperature (20-25°C).

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

8. Pipette 100 µl of conjugate into each well, except H1 and H2.

9. Seal the microtiter plate and shake the plate for a few seconds on a microtiter plate shaker.

10. Incubate for 30 minutes in the dark at room temperature (20-25°C).

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

12. Pipette 100 µl of substrate solution into each well.

13. Incubate 30 minutes at room temperature (20ºC - 25ºC).

14. Add 100 µl of stop solution into each well.

15. Read the absorbance values immediately at 450 nm.

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11. INTERPRETATION OF RESULTS

Subtract the mean optical density (O.D.) of the wells H1 and H2 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 (wells B1 and B2) and multiplied by 100. The zero standard is thus made equal to 100% (maximal absorbance) and the other O.D. values are quoted in percentages of the maximal absorbance.

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

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

Alternative for calibration curve:
The value of absorption (logit) calculation of the standards are plotted on Y-axis versus the analyte equivalent concentration on a logarithmic X-axis

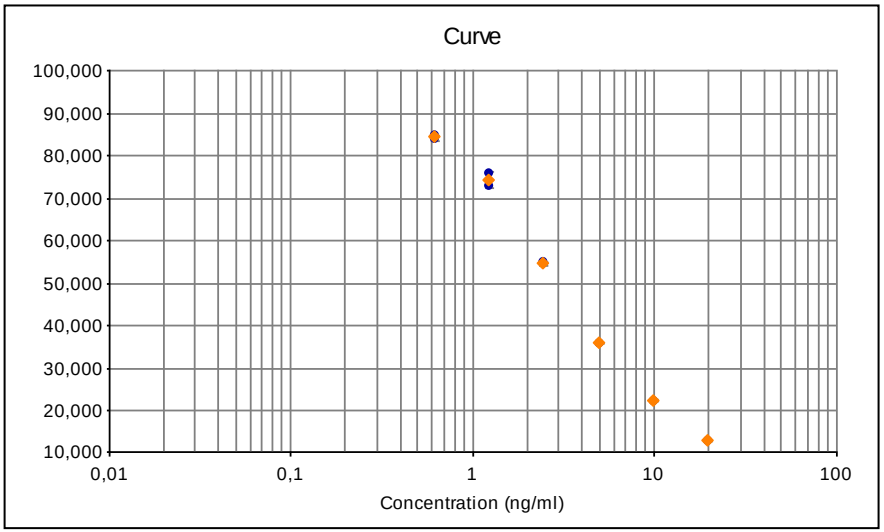

Figure 1 : Example of a calibration curve

Calculation factors

To obtain the TTX concentration in a sample (ng/g), the TTX concentration read from the calibration curve has to be multiplied by a factor 20. This factor is applicable for the sample preparation of fish, as well for shellfish.

12. LITERATURE

European Food Safety Authority (EFSA). Risks for public health related to the presence of tetrodotoxin (TTX) and TTX analogues in marine bivalves and gastropods. *EFSA Journal* 2017, 15(4):4752.

Lago J., Rodríguez L.P., Blanco L., Vieites J.M. and Cabado A.G. Tetrodotoxin, an extremely potent marine neurotoxin: distribution, toxicity, origin and therapeutical uses. *Mar. Drugs* 2015, 13, 6384-6406.

13. ORDERING INFORMATION

For ordering the Tetrodotoxin ELISA, please use catalogue code 5191TTX Tetrodotoxin ELISA.

14. REVISION HISTORY

Not applicable

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