

OXYTETRACYCLINE ELISA

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A competitive enzyme immunoassay for
screening and quantitative analysis of
oxytetracycline in various matrices

EUROPROXIMA OXYTETRACYCLINE ELISA

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TABLE OF CONTENTS

	PAGE:
Brief Information.....	2
1. Introduction	2
2. Principle of the oxytetracycline ELISA.....	3
3. Specificity and Sensitivity	3
4. Handling and Storage	4
5. Kit contents	5
6. Equipment and materials required but not provided.....	6
7. Precautions.....	6
8. Sample preparations	7
9. Preparation of reagents	7
10. Assay Procedure	9
11. Interpretation of results.....	10
12. Literature.....	11
13. Ordering information	11
14. Revision history	11

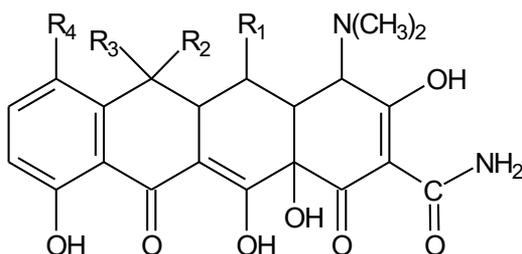
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BRIEF INFORMATION

The oxytetracycline ELISA is a competitive enzyme immunoassay for measurement of the concentration of a group of tetracycline antibiotics in various matrices.

The test is based on antibodies directed against oxytetracyclines. 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 oxytetracyclines from different matrices are included in the kit manual.

1. INTRODUCTION



	R1	R2	R3	R4
Tetracycline	H	OH	CH ₃	H
Oxytetracycline	OH	OH	CH ₃	H
Chlortetracycline	H	OH	CH ₃	Cl
Doxycycline	OH	H	CH ₃	H

Tetracyclines are a group of antibiotics derived from *Streptomyces* spp. with a broad spectrum activity against Gram-negative and Gram-positive aerobic and anaerobic bacteria. Because of their broad spectrum activity, low toxicity profile and low cost, tetracyclines are often used as feed additives for food-producing animals (including honeybees) and in aquaculture. The most commonly used tetracyclines in veterinary medicine are tetracycline (TC), oxytetracycline (OTC), chlortetracycline (CTC) and doxycycline (DC).

Tetracyclines bind to the 30S subunit of microbial ribosomes. They inhibit protein synthesis by blocking the attachment of aminoacyl-tRNA to the A site on the ribosome. In this way, introduction of new amino acids to the nascent peptide chain is prevented. By inhibiting protein synthesis TCs cause cell death of the bacterial cell. The action of tetracyclines is reversible upon withdrawal of the drug.

Residues in food of animal origin may be found, often because of improper observance of withdrawal times. The EU has set maximum residue limits (MRLs) for TC, OTC, CTC and DC: 100 µg/kg in muscle and in milk, 200 µg/kg in egg, 300 µg/kg in liver and 600 µg/kg in kidney. For TC, OTC and CTC these MRLs are expressed as the sum of the parent drug and its 4-epimer, whereas for DC only the parent compound is included in the MRL.

2. PRINCIPLE OF THE OXYTETRACYCLINE ELISA

The microtiter plate based oxytetracycline ELISA consists of one plate (12 strips, 8 wells each) pre-coated with a generic tetracycline antibody. Horseradish peroxidase labeled tetracycline (tetracycline-HRP conjugate), oxytetracycline (standard solution or sample) are added to the pre-coated wells. Oxytetracycline and the tetracycline-HRP conjugate compete for the specific antibody binding sites (competitive enzyme immunoassay).

After an incubation step of 1 hour, the non-bound reagents are removed in a washing step. The amount of bound tetracycline-HRP conjugate is visualized by the addition of a substrate/chromogen solution (tetramethylbenzidine, TMB). Bound tetracycline-HRP conjugate transforms the colourless 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. The optical density is inversely proportional to the tetracycline concentration in the sample.

3. SPECIFICITY AND SENSITIVITY

The oxytetracycline ELISA utilizes antibodies raised in mouse against protein conjugated tetracycline. The reactivity pattern of the antibody is:

Cross- reactivity:	Oxytetracycline	100%
	Tetracycline	133%
	Chlortetracycline	138%
	Doxycycline	54%

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	Procedure	LOD (ppb)
Honey	8.1	5
Shrimps/fish	8.2	2

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 (2°C to 8°C) 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:

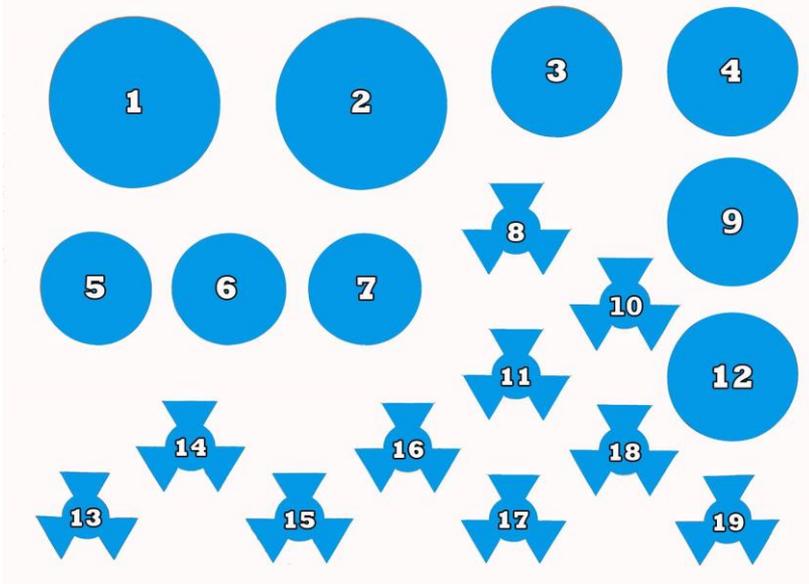
- A blue colouring of the chromogen solution before transferring it into the wells.
- A weak or no colour reaction in the zero standard wells ($E_{450nm} < 0.8$).

5. KIT CONTENTS

Manual

One sealed (96-wells) microtiter plate (12 strips, 8 wells each), coated with specific Tetracyclines antibody. 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. **Conjugate** (lyophilized, blue cap)
6. **Oxytetracycline standard** (lyophilized 2.5 ng/ml, black cap)
7. **Oxytetracycline standard** (lyophilized 2.5 ng/ml, black cap)
8. Not in use
9. Not in use
10. Not in use
11. Not in use
12. Not in use
13. **Oxytetracycline standard** (lyophilized 100 ng/ml, black cap)
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

- 4 ml glass tubes
- 15 ml tubes with screw cap (polypropylene)
- 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
- Methanol 100%
- Sodium Dibasic (Na_2HPO_4)
- Trisodium Citrate Dihydrate ($\text{C}_6\text{H}_5\text{O}_7\text{Na}_3 \cdot 2\text{H}_2\text{O}$)
- 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 +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.

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8. SAMPLE PREPARATION

8.1 Honey

- Weigh 1 g sample, add 1.5 ml 80% v/v methanol/distilled water (80:20)
- If necessary, heat the honey 15 minutes, 50°C, vortex, mix head-over-head for 15 minutes
- Centrifuge at 2000 x g for 5 minutes (20°C - 25°C)
- Dilute 50 µl of this solution with 350 µl dilution buffer, vortex
- Use 50 µl of this solution in the ELISA test

8.2 Shrimps/fish

- Weigh 1 g of homogenized shrimp or fish into a 15 ml plastic tube
- Add 3 ml of McIlvain Buffer
- Vortex rigorously and mix the samples head-over-head for 10 minutes
- Centrifuge the samples at 2000 x g for 10 minutes
- Pipette 50 µl of the supernatant into a new tube and add 200 µl of dilution buffer
- Use 50 µl of this solution 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.

Dilution buffer

The dilution buffer 4x concentrated. Dilute the buffer 1:4 (1 ml buffer + 3 ml distilled water) before use.

Sample dilution buffer

Sample dilution buffer is not provided in the kit. Prepare this buffer as follows: Take 18 ml dilution buffer, add 2 ml 100% methanol, mix and store this buffer at 4°C until use.

Standard 2.5 ng/ml

Prepare a dilution range of oxytetracycline standards. Add 2 ml of sample dilution buffer to the oxytetracycline standard and mix. This solution contains 2.5 ng oxytetracycline per ml. Pipette 0.25 ml of this solution into a clean tube and add 0.25 ml of sample dilution buffer. Continue to make a dilution range of 1.25, 0.625, 0.3125, 0.156 and 0.078 ng/ml.

Standard solution (100 ng/ml)

Reconstitute the vial of lyophilized oxytetracycline standard with 1 ml of sample dilution buffer, mix thoroughly and keep in the dark until use.

To prepare standards in the appropriate matrix or to prepare spikes use the standard solution containing 100 ng oxytetracycline per ml. Dilute the standard solution in the appropriate matrix to make a dilution range of 2.5, 1.25, 0.625, 0.3125, 0.156, 0.078 ng/ml.

Conjugate

Reconstitute the vial of lyophilized conjugate with 6 ml of dilution buffer, mix thoroughly and keep in the dark until use.

Rinsing buffer

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

Substrate/chromogen solution

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

Mcllvain Buffer for shrimps/fish (8.3)

Prepare 0.2 M Sodium Dibasic solution:

Na_2HPO_4 28.4 g

Distilled water up to 1 liter

Prepare 0.1 M Trisodium Citrate Dihydrate solution:

$\text{C}_6\text{H}_5\text{O}_7\text{Na}_3 \cdot 2\text{H}_2\text{O}$ 29.4 g

Distilled water up to 1 liter

Mix above solutions 1 : 1, check pH; adjust pH to 7.0 with HCl

Dilute 1 : 1 with methanol before use

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 μ l) 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 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 and prepare reagents according to Chapter 9.
2. Pipette 100 μ l of the sample dilution buffer in duplicate (wells H1, H2, blank).
Pipette 50 μ l of the sample dilution buffer (zero standard, Bmax) in duplicate (wells A1, A2).
Pipette 50 μ l of each of the standard solutions in duplicate (wells B1,2 to G1,2 i.e. 0.078, 0.156, 0.3125, 0.625, 1.25 and 2.5 ng/ml).
3. Pipette 50 μ l of each sample solution in duplicate into the remaining wells of the microtiter plate.
4. Pipette 50 μ l of conjugate to all wells, except H1 and H2.
5. Seal the microtiter plate and shake the plate for a few seconds on a microtiter plate shaker.

6. Incubate for 1 hour in the dark at 20°C to 25°C.
7. Discard the solution from the microtiter plate and wash 3 times with rinsing buffer.
8. Pipette 100 µl of substrate solution into each well.
9. Incubate 30 minutes in the dark at 20°C to 25°C.
10. Add 100 µl of stop solution to each well.
11. Read the absorbance values immediately at 450 nm.

11. INTERPRETATION OF RESULTS

In general, the O.D. values of the samples, that are measured in this ELISA test, are converted into corresponding concentrations in the following way:

The mean optical density (O.D.) value of the blank wells is subtracted from the individual O.D. of the wells containing the standards and the samples. Then these O.D. values of the standards and the samples (mean values of the duplicates) are divided by the mean O.D. value of the zero standard 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.

The values calculated for the standards are entered in a semi-logarithmic plot against the concentration of the analyte.

Alternative for calibration curve:

The values of absorption (logit) calculation of the standards are plotted on Y-axis versus the analyte equivalent concentration on a logarithmic X-axis

As customer-friendly options R-Biopharm offers 2 software programs that can be used for the interpretation of the ELISA test:

- RIDASOFT®Win.NET Food & Feed (art. nr. Z9996FF). In this software program all R-Biopharm ELISA tests (including EuroProxima) are preprogrammed in the database. A list of compatible readers is available on request.
- Simplefit. This Excel based program is developed to calculate the results of all EuroProxima tests.

8.1 Honey

The oxytetracycline equivalents, as read from the standard curve, have to be multiplied by a factor 20 to obtain the oxytetracycline content in honey samples.

8.2 Shrimps/fish

The oxytetracycline equivalents, as read from the standard curve, have to be multiplied by a factor 20 to obtain the oxytetracycline in shrimps or fish samples.

12. LITERATURE

Mechanism of Action of Tetracyclines.

<http://pharmaxchange.info/press/2011/05/mechanism-of-action-of-tetracyclines/>

Council Regulation (EEC) No 2377/90 of 26 June 1990. Off. J. Eur. Commun. 1990, **L224**, 1-8.

Commission Regulation (EC) No 508/1999 of 4 March 1999. Off. J. Eur. Commun. 1999, **L60**, 16-52.

13. ORDERING INFORMATION

For ordering the oxytetracycline ELISA kit, please use cat. code 5091OTC.

14. REVISION HISTORY

Chapter 11 has been updated.