OXYTETRACYCLINE ELISA

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A competitive enzyme immunoassay for screening and quantitative analysis of oxytetracyclines

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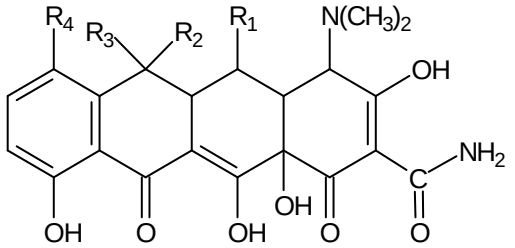
BRIEF INFORMATION

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

Tetracylines 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 [1].
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 [2,3].

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- reactvity:	Oxytetracycline	100%
	Tetracycline	133%
	Chlortetracycline	138%
	Doxycycline	54%

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

Matrix	Procedure	LOD ppb
Honey	8.1	5
Shrimps/fish	8.2	2

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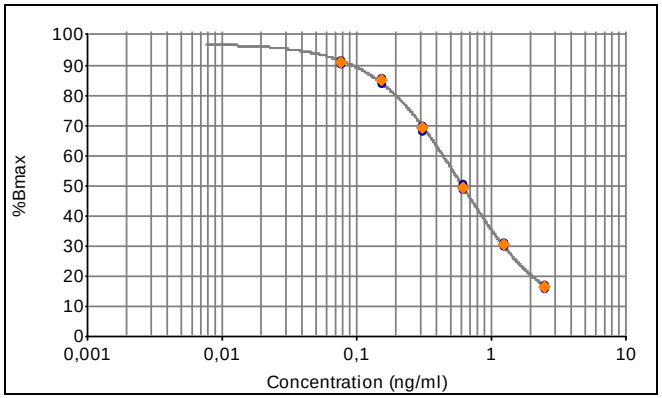


Figure 1 : Example of a calibration curve

The amount of oxytetracyclines in the samples is expressed as oxytetracycline equivalents. The oxytetracycline equivalents in the samples (ng/ml) corresponding to the % maximal absorbance of each extract can be read from the calibration curve.

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

1. Mechanism of Action of Tetracyclines. http://pharmaxchange.info/press/2011/05/mechanism-of-action-of-tetracyclines/
2. Council Regulation (EEC) No 2377/90 of 26 June 1990. Off. J. Eur. Commun. 1990, **L224**, 1-8.
3. 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. LAST MUTATIONS

Standard solution 100 ng/ml is added.

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.

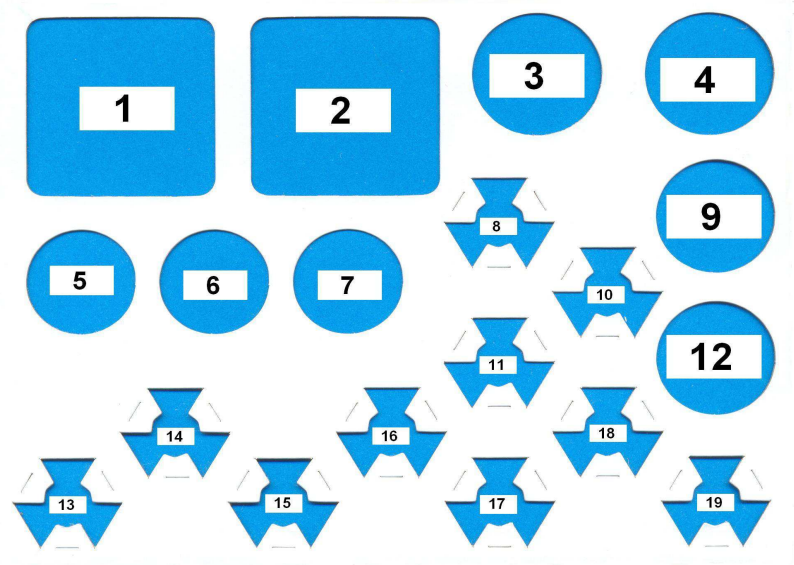
Degeneration of the reagents may have occurred when the following phenomena are observed:
- A blue colouring of the chromogen solution before transferring it into the wells.
- A weak or absent colour reaction of the maximum binding (zero standard) (E450nm < 0.8).

5. KIT CONTENTS

Manual

One sealed (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. D**ilution 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. Incubate for 1 hour in the dark at 20ºC to 25ºC.
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 20ºC to 25ºC.
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 sample dilution buffer*/Bmax (wells A1 and A2) and multiplied by 100. The sample dilution buffer* /Bmax is thus made equal to 100% (maximal absorbance) and the other O.D. values are quoted in percentages of the maximal absorbance.

O.D. standard (or sample)
----------x 100% = % maximal absorbance
O.D. sample dilution buffer

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

Alternative for calibration curve:
The absorbtion 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.

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 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 (Sample preparation) and prepare reagents according to Chapter 9 (Preparation of reagents).

2. Pipette 100 µl of the sample dilution buffer* in duplicate (wells H1, H2, blank).
   Pipette 50 µl of the sample dilution buffer* (zero standard, Bmax) in duplicate (wells A1, A2).
   Pipette 50 µl 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 µl of each sample solution in duplicate into the remaining wells of the microtiter plate.

4. Pipette 50 µl 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. EQUIPMENT AND MATERIALS REQUIRED BUT NOT PROVIDED

- 4 ml glass tubes
- 15 ml tubes with screw cap (Greiner, 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 ($C_6H_5O_7Na_3 \bullet 2H_2O$)

7. PRECAUTIONS

- Oxytetracyclines are toxic compounds. Avoid contact with mouth and skin. Be aware that oxytetracyclines are not inhaled.
- 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 +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 Honey
- Weigh 1 g sample, add 1.5 ml 80% v/v methanol/distilled water (80:20)
- If necessary, heat the honey 15 minutes, 50ºC, vortex, mix head-over-head for 15 minutes
- Centrifuge at 2000 x g for 5 minutes (20ºC - 25ºC)
- 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 in the ELISA

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 2ºC to 8ºC for use in subsequent assays. Retain also the strip holder.

Dilution buffer
The dilution buffer chapter 5 no.1 is 4x concentrated. Dilute the buffer 1:4 (1 ml buffer + 3 ml distilled water) before use. This buffer is for dissolving conjugate and to prepare the sample dilution buffer*.

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 4ºC until use.

McIlvain 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:
$C_6H_5O_7Na_3 \cdot 2H_2O$ 29.4 g
Distilled water up to 1 liter

Buffer components can be stored at 2ºC to 8ºC. Final buffer mix has to be prepared freshly before use.

Mix above solutions 1 : 1, check pH; adjust pH to 7.0 with HCl
Dilute 1 : 1 with Methanol before 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.
For prolonged storage: freeze aliquots at -20ºC.

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 (chapter 5 no.1), mix thoroughly and keep in the dark until use.
For prolonged storage: freeze aliquots at -20ºC.

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).

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.