TETRACYCLINE ELISA

5091TC[7]04.20

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

EUROPROXIMA TETRACYCLINE ELISA

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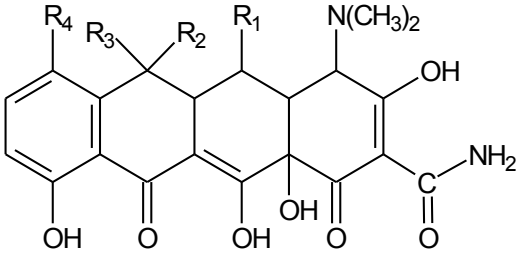
BRIEF INFORMATION

The tetracycline ELISA is a competitive enzyme immunoassay for measurement of the concentration of a group of tetracycline antibiotics in various matrices. 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 ELISA kit contains all the reagents, including standards, required to perform the test. However, no reagents for sample preparation are included.

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.
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 TETRACYCLINE ELISA

The microtiter plate based tetracycline ELISA consists of one plate (12 strips, 8 wells each) pre-coated with a specific antibody to tetracycline. Horseradish peroxidase labeled tetracycline (tetracycline-HRP conjugate), tetracycline (standard solution or sample) are added to the pre-coated wells. Tetracycline 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 tetracycline ELISA utilizes antibodies raised in mouse against protein conjugated tetracycline. The reactivity pattern of the antibody is:

Cross- reactions:	Tetracycline	100%
	4-epitetracycline	87%
	Rolitetracycline	67%
	4-epioxytetracycline	52%
	Oxytetracycline	52%
	Chlortetracycline	51%
	Demeclocycline	41%
	Doxycycline	23%
	4-epichlortetracycline	20%
	Methacycline	11%

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)
milk	8.1	0.4
honey	8.2	1.7
tissue/liver	8.3	2.9
shrimps	8.4	1.3
Egg	8.4	4.0
Butter	8.5	2.1

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:

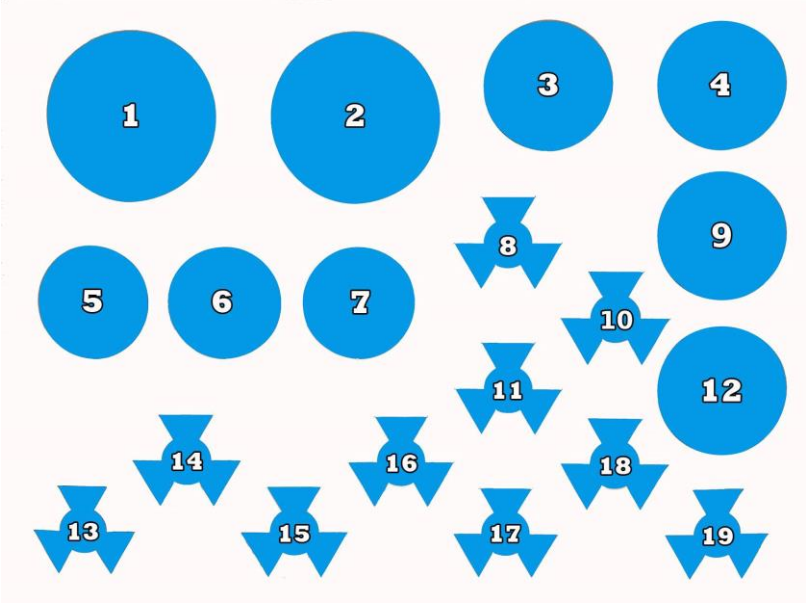
- A blue colouring of the chromogen solution before transferring it into the wells.
- A weak or no colour reaction in the zero standard wells (E450nm < 0.8).

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. **Tetracycline standard** (lyophilized 2 ng/ml, black cap)
7. **Tetracycline standard** (lyophilized 1000 ng/ml, black cap)
8. Not in use
9. **MTC buffer (**14 ml, ready-to-use)
10. Not in use
11. Not in use
12. Not in 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

- 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
- Pasteur pipette
- Methanol 100%
- Sodium Dibasic (Na_2HPO_4)
- Trisodium Citrate Dihydrate ($C_6H_5O_7Na_3 \cdot 2H_2O$)
- Distilled water
- N-Hexane

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 Milk

For undiluted defatted milk samples the matrix effect is a critical factor. To avoid fat residues in the sample, the manual should be strictly followed. The pH of the sample is another potential pitfall. Sour milk disturbs the ELISA, neutralisation of the pH is essential.

Milk powder

To milk powder water has to be added to obtain “milk”.
E.g. to 1 g milk powder an amount of 9 ml distilled water is added.

Procedure:

- Centrifuge cold milk samples at 2000 x g for 15 minutes at 4ºC
- Remove the upper fat layer using a spatula
- Pipette 0.75 ml defatted milk in a clean tube, add 0.25 ml MTC buffer (chapter 5, no. 9), vortex rigorously
- Pipette 100 µl of the diluted milk in 200 µl sample dilution buffer (see chapter 9), vortex
- Use 50 µl of this solution in the ELISA test

8.2 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 sample dilution buffer (see chapter 9), vortex
- Use 50 µl of this solution in the ELISA test

8.3 Tissue/liver

- Weigh 1 g finely cut and subsequently homogenized tissue in a clean tube
- Add 0.5 ml distilled water, add 1.5 ml 100% methanol, 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 sample dilution buffer (see chapter 9), vortex
- Use 50 µl of this solution in the ELISA test

8.4 Shrimps/egg

- Weigh 1 g of homogenized shrimp or egg into a 15 ml plastic tube
- Add 3 ml of McIlvain Buffer pH 7
- 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

8.5 Butter
- Weigh 1 g butter into a 10 ml centrifugal vial
- Melt the butter in a water bath at approximately 40°C
- Add 1 ml n-hexane and mix vigorously on a vortex for 1 minute
- Add 1 ml Methanol 20% (v/v; methanol/water)
- Mix vigorously on a vortex for 10 seconds and mix the samples head-over-head for 10 minutes
- Centrifuge the samples at 2000 x g for 10 minutes at 4ºC
- Remove the upper hexane layer carefully with a Pasteur pipette
- Add 1 ml of n-hexane and mix vigorously on a vortex for 1 minute
- Centrifuge the samples at 2000 x g for 10 minutes at 4ºC
- Transfer 1 ml of the layer underneath (aqueous) into a 1.5 ml vial and keep the vial in a freezer for 5 minutes at -20°C
- Centrifuge the samples at 2000 x g for 5 minutes (20ºC - 25ºC)
- Dilute 50 µl of the aqueous phase with 800 µl sample dilution buffer (see chapter 9)
- Use 50 µl of this solution in the ELISA

9. PREPARATION OF REAGENTS

Before beginning the test, the reagents should be brought up 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 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 is 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 4ºC until use.

Standard 2 ng/ml
Prepare a dilution range of tetracycline standards. Add 2 ml of sample dilution buffer to the tetracycline standard and mix. This solution contains 2 ng tetracycline 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 1.0, 0.5, 0.25, 0.125 and 0.0625 ng/ml.

Standard 1000 ng/ml
This standard is for spiking.
Add 1 ml of sample dilution buffer to the tetracycline standard and mix. This solution contains 1000 ng tetracycline per ml.

Conjugate
Reconstitute the vial of lyophilized conjugate (tetracycline-HRP) 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 +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.

McIlvain Buffer
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

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 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 and prepare reagents according to Chapter 9.

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. 0.0625, 0.125, 0.25, 0.5, 1.0 and 2.0 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 (tetracycline-HRP) 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 room temperature (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 room temperature (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.

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

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

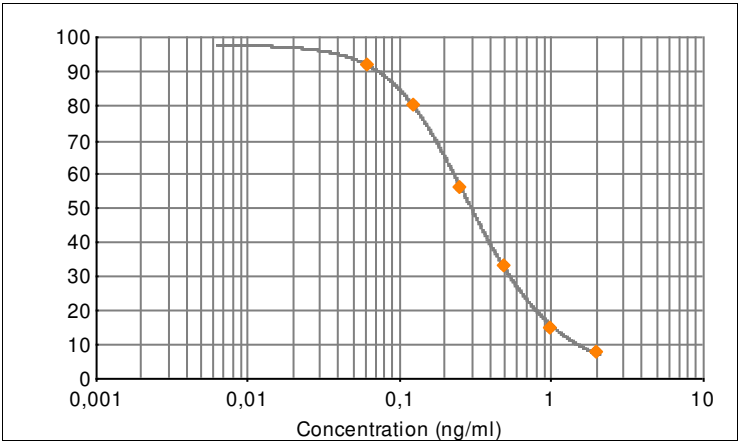


Figure 1 : Example of a calibration curve

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

8.1 Milk and milk powder
To obtain the tetracycline content in milk or milk powder samples, the calculated tetracycline concentration has to be multiplied by a factor 4.

8.2 Honey
To obtain the tetracycline content in honey samples, the calculated tetracycline concentration has to be multiplied by a factor 20.

8.3 Tissue/liver
To obtain the tetracycline content in tissue or liver samples, the calculated tetracycline concentration has to be multiplied by a factor 24.

8.4 Shrimps/egg
To obtain the tetracycline content in shrimps or egg samples, the calculated tetracycline concentration has to be multiplied by a factor 20.

8.5 Butter
To obtain the tetracycline content in butter samples, the calculated tetracycline concentration has to be multiplied by a factor 34.

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 tetracycline ELISA kit, please use cat. code 5091TC.

14. REVISION HISTORY

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

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