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

8.2 Serum and urine samples, direct
To obtain the tylosin content in serum and urine samples, the calculated tylosin concentration has to be multiplied by a factor 4.

8.3 Tissue, honey, egg and feed samples, direct
To obtain the tylosin content in tissue, honey, egg and feed samples, the calculated tylosin concentration has to be multiplied by a factor 24.

8.4 Tissue, honey and feed samples (SPE)
To obtain the tylosin content in tissue, honey and feed samples, the calculated tylosin concentration has to be multiplied by a factor 4.

12. LITERATURE

1. Council Directive 70/524/EEC of 23 November 1970 concerning additives in feeding stuffs. *Official Journal of the European Communities L270: 1-34.*
2. Council Regulation 2821/98 of 17 December 1998 amending, as regards withdrawal of the authorisation of certain antibiotics, Directive 70/524/EEC concerning additives in feeding stuffs. *Official Journal of the European Communities L351: 4-8.*
3. Commission Regulation 2788/98 of 22 December 1998 amending Council Directive 70/524/EEC concerning additives in feeding stuffs as regards the withdrawal of authorisation for certain growth promoters. *Official Journal of the European Communities L347: 31-32.*

13. ORDERING INFORMATION

For ordering the Tylosin ELISA kit, please use cat. code 5151TYL.

14. LAST MUTATIONS

Egg sample is added.
Pipette schedule of the standard curve is adapted.

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Beijerinckweg 18
NL 6827 BN Arnhem
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TYLOSIN ELISA

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

Tylosin ELISA

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

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6. Pipette 100 µl of substrate solution into each well.
7. Incubate for 15 minutes in the dark at room temperature (20°C to 25°C).
8. Pipette 100 µl of stop solution to each well.
9. Read the absorbance values immediately at 450 nm.

11. INTERPRETATION OF RESULTS

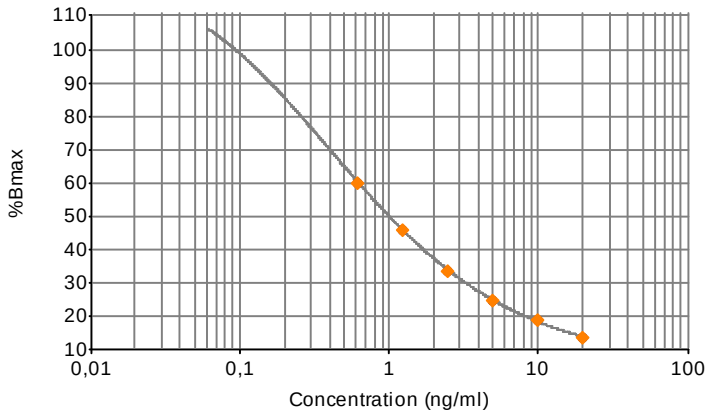
Subtract the mean optical density (O.D.) of the wells H1 and H2 (Blank) from the individual O.D. of the wells containing the standards and the samples.

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.

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

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



10. ASSAY PROCEDURE

Rinsing protocol

In ELISAs, 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 rinsing 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 (Preparations of reagents).
   The microtiter plate is ready-to-use.

2. Pipette 100 µl of zero standard in duplicate (wells H1,H2, blank).
   Pipette 50 µl of zero standard in duplicate (wells A1,A2, Bmax).
   Pipette 50 µl of each of the Tylosin standard solutions in duplicate (wells ng/ml).
   Pipette 50 µl of each of the sample solution in duplicate into the remaining wells of the microtiter plate.

3. Pipette 50 µl of conjugate solution to all wells, except wells H1, H2.

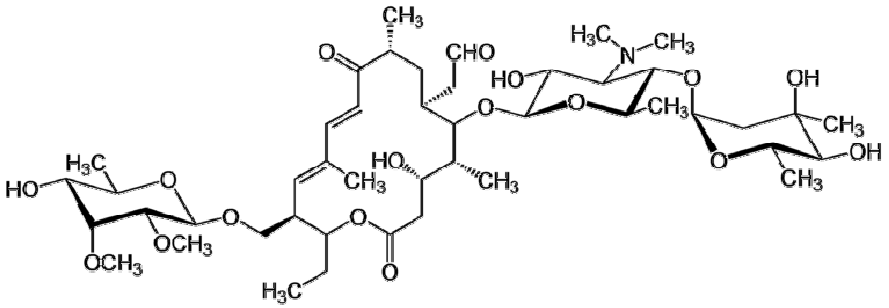
4. Incubate 1 hour at room temperature (20°C to 25°C).

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

BRIEF INFORMATION

The tylosin ELISA is a competitive enzyme immunoassay for measurement of the concentration of tylosin. 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 analyzed.
The ELISA kit contains all reagents to perform the assay. Reagents for sample preparation are not included in the kit.

1. INTRODUCTION



Chemical structure of tylosin

Antibacterial drugs intended to be used as growth promoters are added to the feed of entire herds and flocks at sub-therapeutic levels over an extended period of time. These performance enhancing antibacterial growth promoters are regulated by Council Directive 70/524/EEC [1] as zootechnical feed additives and specified tolerances for their inclusion are given in the annex of that document.
Whenever drug preparations are administered to food-producing animals, residues thereof in edible tissues, milk or eggs are likely. Residues of antibacterial drugs in food could lead to allergic reactions but the greatest threat is the development of resistant strains of bacteria which could lead to an improper response to normal drug treatment in humans. For these reasons the European Commission decided to ban some of the regulated growth promoters. With Council Regulation 2821/98 [2] zinc bacitracin, spiramycin, tylosin and virginiamycin, and with Commission Regulation 2788/98 [3] olaquindox, were banned from animal feed.

2. PRINCIPLE OF THE TYLOSIN ELISA

The microtiter plate based tylosin ELISA kit consists of one precoated plate (12 strips, 8 wells each). Antibody, Horseradish peroxidase (-HRP) labelled tylosin and standard solution or sample are added to the wells. Free tylosin from the samples or standards and tylosin-HRP conjugate compete for the specific antibody binding sites (competitive enzyme immunoassay).
After an incubation time of 60 minutes, the non-bound reagents are removed in a washing step. The amount of bound tylosin-HRP conjugate is visualized by the addition of substrate/chromogen solution (H_2O_2/TMB). Bound tylosin 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 tylosin concentration in the sample.

3. SPECIFICITY AND SENSITIVITY

The tylosin ELISA utilizes antibodies raised in rabbits against protein conjugated Tylosin. The reactively pattern of this antibody is:

Cross-reactivity:	Tylosin A	100%
	Tylosin B (Desmycosin)	36%

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

Matrix	Procedure	LOD
Milk	8.1	2.5
Honey	8.3	15
Honey	8.4	2.5
Egg	8.3	2.5
Tissue	8.3	15
Tissue	8.4	2.5
Serum	8.2	2.5
Urine	8.2	2.5
Feed	8.3	15
Feed	8.4	2.5

N.B. This ELISA can be used at different levels of sensitivity. Samples can be diluted with a factor. The LOD-values shown in the table above refer to measuring ranges 2.5-80 ng/ml and 15-480 ng/ml. By further diluting the samples 1 : 10, the measuring ranges can be extended from 25-800 ng/ml and 150-4800 ng/ml.

- Washing procedure
  - Add 4 ml methanol/distilled water (10:90 = v:v)
  - Add 4 ml methanol/distilled water (20:80 = v:v), allow disk to become dry.

- Elution of tylosin
  - Pipette 1 ml 100% methanol
  - Pipette 1 ml 100% acetonitrile
- Let the cartridge run dry for 2 minutes
- Evaporate the eluent to dryness under a mild stream of nitrogen, 50°C - 60°C
- The residue is dissolved in 1 ml of water/methanol*, vortex, dilute 20 µl of the dissolved residue with 180 µl sample dilution buffer
- Vortex
- Use 50 µl of the diluted sample in the ELISA

* Water/methanol (10%)
180 ml distilled water with 20 ml methanol (p.a. 100%)

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 +2°C to +8°C. 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.

Conjugate
The conjugate delivered is 100x concentrated. Spin down the conjugate in the vial by a short centrifugation step (1 minute, 1000 x g). Add 10 µl of the concentrated conjugate solution to 990 µl diluent for conjugate. Per 2 x 8 wells 800 µl is required. Store unused concentrated conjugate at 2°C - 8°C.

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

8. SAMPLE PREPARATION

8.1 Milk samples
- Centrifuge cold milk samples for 5 minutes at 2000 x g at 4°C
- Remove the upper fat layer using a spatula
- Dilute the defatted milk 4 times (50 µl defatted milk + 150 µl sample dilution buffer).
- Vortex
- Use 50 µl of the diluted sample in the ELISA

8.2 Serum and urine samples direct
- Centrifuge the samples for 5 minutes at 2000 x g
- Dilute the supernatant 4 times (50 µl sample + 150 µ sample dilution buffer)
- Vortex
- Use 50 µl of the diluted sample in the ELISA

8.3 Tissue, honey, egg and feed samples direct
- Weigh 1 g finely and subsequently homogenized sample into a 15 ml polypropylene tube
- Add 5 ml water/methanol*
- Vortex
- Mix well on a vortex followed by mixing head over head for at least 15 minutes
- Centrifuge 5 minutes at 2000 x g
- Dilute the clear supernatant 4 times with sample dilution buffer (50 µl supernatant + 150 µl sample dilution buffer)
- Vortex
- Use 50 µl of the diluted sample in the ELISA

8.4 Tissue, honey and feed samples (SPE)
- Weigh in 5 g honey into a clean tube
- Add 20 ml 0.1 M Sodium phosphate buffer pH 7.5 – 8.0 (Chapter 6)
- Mix head-over-head for 15 minutes at room temperature
- Centrifuge 15 minutes at 2000 x g at room temperature
- Use 12.5 ml of the supernatant for further purification

Solid phase extraction (SPE)
- Use Oasis HLB 3 cc extraction cartridges
- Conditioning of the cartridges:
  - Add 5 ml of 100% methanol (flow 1 ml/min.)
  - Add 5 ml of distilled water (flow 1 ml/min.)

Note: Keep the sorbent wet during conditioning and prior to sample addition. If the sorbent runs dry, repeat the conditioning procedure.

- Carefully transfer the 12.5 ml supernatant onto the activated cartridge. Pass the sample through the disk using vacuum; flow 1 ml/min.

4. HANDLING AND STORAGE

- Store the kit 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 (see kit label) has passed, quality claims are not accepted.
- Before opening the sealed plate, the plate should be at ambient temperature in order to avoid condensation in the ELISA.
- Dilute the kit components immediately before use, but after the components are at ambient temperature.
- The substrate/chromogen solution 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 substrate/chromogen solution should be avoided.

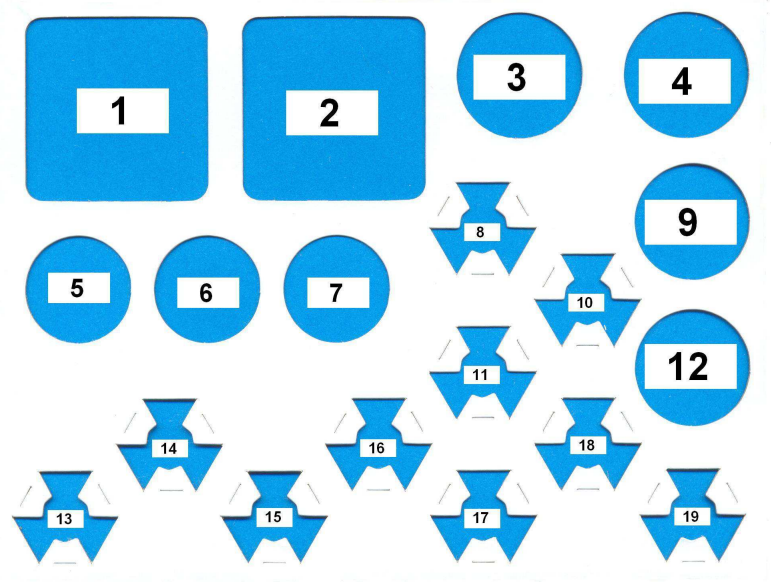
Degeneration of the reagents may have occurred when the following phenomena are observed:
- A blue colouring of the substrate/chromogen solution before transferring it into the wells.
- A weak or absent colour reaction of the zero standard (Bmax, E450nm < 0.8).

5. KIT CONTENTS

Manual

One sealed (96-wells) microtiter plate (12 strips, 8 wells each), coated with antibody. Plate is ready-to-use.

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



1. **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** (15 ml, Ready-to-use)
5. not in use
6. not in use
7. not in use
8. **Conjugate solution** (150 µl, 100x concentrated)
9. **Diluent (for conjugate)** (10 ml)
10. not in use
11. **Tylosin stock solution** (1 ml, 100 ng/ml)
12. not in use
13. **Zero standard solution** (2 ml, Ready-to-use)
14. **Standard solution 0.625 ng/ml** (1 ml, Ready-to-use)
15. **Standard solution 1.25 ng/ml** (1 ml, Ready-to-use)
16. **Standard solution 2.5 ng/ml** (1 ml, Ready-to-use)
17. **Standard solution 5 ng/ml** (1 ml, Ready-to-use)
18. **Standard solution 10 ng/ml** (1 ml, Ready-to-use)
19. **Standard solution 20 ng/ml** (1 ml. Ready-to-use)

6. EQUIPMENT AND MATERIALS REQUIRED BUT NOT PROVIDED

- Evaporation equipment (sample extraction honey)
- Scales and weighing vessels
- Gloves
- Fume hood
- Homogeniser (vortex, mixer)
- Centrifuge (2000 x g)
- Automated microplate washer or 8-channel micropipette 100 – 300 µl
- Microtiter plate shaker
- Microtiter plate reader with 450 nm filter
- Glass tubes (10 – 15 ml)
- Micropipettes 20 – 200 µl, 100 – 1000 µl
- 8-Channel micropipette 100 – 300 µl
- Multi-stepper pipette with 2.5 ml combitips
- Distilled water
- Methanol 100%
- Acetonitrile for HPLC gradient grade
- Oasis HLB 3cc cartridges (WAT094226)
- 0.1 M Sodium phosphate buffer pH 7.5 – 8.0:
  Solution A: 6.9 g NaH_2PO_4. H_2O/250 ml distilled water
  Solution B: 10.6 g Na_2HPO_4. $2H_2O$/250 ml distilled water
  Take 40 ml solution A and 210 ml solution B dilute with 250 ml distilled water

7. PRECAUTIONS

- Tylosin is toxic compound. Avoid contact with mouth and skin. Be aware that Tylosin is 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; take care when handling the substrate.
- 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, 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.