VIRGINIAMYCIN ELISA

5151VIG[6]06.15

A competitive enzyme immunoassay
for quantitative analysis of Virginiamycin in
various matrices

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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 Virginiamycin ELISA kit, please use cat. code 5151VIG.

14. LAST MUTATIONS

Conjugate adapted to 150 µl.
Calibration curve.
Pipette schedule of the standard curve is adapted.
Updated “lay out”

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

7. Incubate for 30 minutes in the dark at 20°C to 25°C.

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

9. Immediately after adding the stop solution, read the absorbance values at 450 nm.

11. INTERPRETATION OF RESULTS

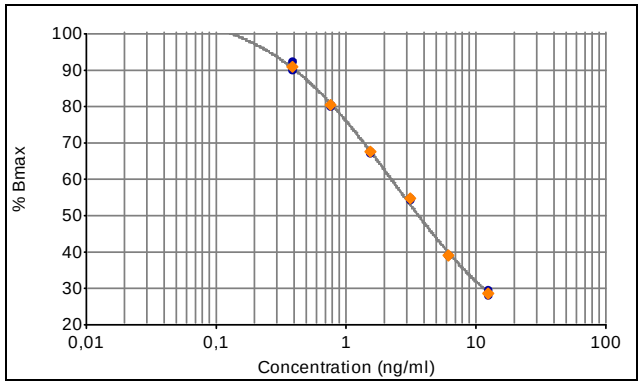
Subtract the mean optical density (O.D.) wells H1 and H2 (Blank) from the individual O.D. of the wells containing the standards and the samples.

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 (% maximal absorbance) calculated for the standards are plotted on the Y-axis versus the analyte equivalent concentration (ng/ml) on a logarithmic X-axis.

Alternative for calibration curve:
The absorption 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.



Example of a calibration curve

BRIEF INFORMATION

The Virginiamycin ELISA (VIG ELISA) is a competitive enzyme immunoassay for screening on the presence of Virginiamycin in urine. The test is based on antibodies directed against virginiamycin.
The ELISA kit contains a 96 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 virginiamycin from different samples are included in the kit manual.

1. INTRODUCTION

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 VIRGINIAMYCIN ELISA

The kit is based on a microtiter platef (12 strips, each 8 wells), precoated with specific antibodies (rabbit anti-virginiamycin). Horseradish peroxidase labelled virginiamycin (enzyme conjugate) and virginiamycin standards or samples are added to the precoated wells followed by a single incubation step. Free virginiamycin (in the standard solution or in the sample) and enzyme labelled virginiamycin 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 enzyme conjugate is visualized by the addition of substrate chromogen (tetramethylbenzidine, TMB). Bound enzyme transforms the 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 and is inversely proportional to the virginiamycin concentration in the sample.

3. SPECIFICITY AND SENSITIVITY

In Virginiamycin-ELISA utilizes a specific antibody raised in rabbits against protein conjugated Virginiamycin.

Cross-reactivity: 100%.

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

Matrix	Procedure	LOD ppb
Milk	8.1	8 ng/ml
Feed	8.2	40 ng/ml
Urine	8.3	40 ng/ml

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).

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 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 sample dilution buffer (SDB) in duplicate (wells H1, H2, blank).
   Pipette 50 µl of sample dilution buffer in duplicate (wells A1, A2, Bmax).
   Pipette 50 µl of each of the Virginiamycin 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 in the dark at 20°C to 25°C.

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

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.

Conjugate 150 µl
The conjugate is delivered 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 to 8°C.

Standard solutions
Prepare a dilution range of the virginiamycin standard. Add 2 ml SDB to the vial of virginiamycin standard. This solution contains 12.5 ng/ml. Make a dilution (steps 1:1) resulting in a range of 12.5, 6.25, 3.125, 1.56, 0.78 and 0.39 ng/ml.
Standards should be prepared freshly before use. Three vials of concentrated standard are provided in the kit.

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 required (2 ml of concentrated rinsing buffer + 38 ml of distilled water).

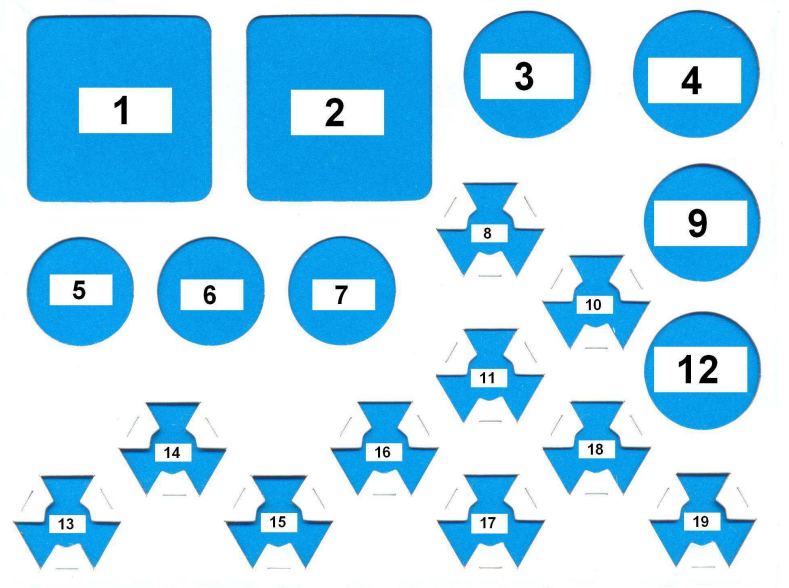
Substrate/chromogen solution
The substrate/chromogen solution (ready-to-use) tends to precipitate at 4°C. Take care that this vial is at room temperature and mix the content well before use. Avoid direct (sun) light.

5. KIT CONTENTS

Manual

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

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



1. **Sample dilution buffer** (SDB. 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. **Standard** (lyophilized, 12.5 ng/ml)
6. **Standard** (lyophilized, 12.5 ng/ml)
7. **Standard** (lyophilized, 12.5 ng/ml)
8. **Conjugate solution** (150 µl, 100x concentrated)
9. **Diluent** (10 ml) for conjugate
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

- Gloves
- Vortex
- 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
- Aluminium foil or parafilm

7. PRECAUTIONS

- Virginiamycin is a toxic compound. Avoid contact with mouth and skin. Be aware that virginiamycin 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; 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 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.

8. SAMPLE PREPARATION

8.1 Milk samples

Note:
The pH of the sample is a potential pitfall. Sour milk disturbs the ELISA, neutralization of the pH is essential. Adjust pH 7 ± 0.5 with 0.1 M NaOH

Sample treatment:
- Centrifuge cold milk samples for 15 minutes at 2000 x g at 4°C.
- Remove the upper fat layer using a spatula.
- Dilute the defatted milk sample 20 times in sample dilution buffer (10 µl defatted milk + 190 µl sample dilution buffer).
- Mix the diluted milk (vortex).
- An aliquot of 50 µl is used in the ELISA test.

8.2 Feed samples
- Grind 10 to 100 gram of feed sample.
- Homogenise 0.5 gram of the grinded feed with 4.5 ml distilled water.
- Vortex and mix 15 minutes head-over-head.
- Centrifuge for 10 minutes at 2000 x g.
- Dilute the supernatant 1:10, 50 µl supernatant with 450 µl sample dilution buffer (chapter 5, no.1).
- An aliquot of 50 µl is used in the ELISA test.

8.3 Urine samples
- Urine samples can be analyzed after a 100 times dilution step in sample dilution buffer (Paragraph 5, no. 1)

Dilution: Add to 10 µl urine 90 µl sample dilution buffer (1:10)
Vortex
Take 50 µl of 1:10 dilution and add 450 µl sample dilution buffer
(Total dilution 1:100)
Vortex

- An aliquot of 50 µl is used in the ELISA test.