BACITRACIN ELISA

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A competitive enzyme immunoassay for screening and quantitative analysis of Bacitracin 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 Bacitracin ELISA kit, please use cat. code 5151BAC.

14. LAST MUTATIONS

Assay protocol 2. Sample dilution buffer revised to zero standard.
Updated lay-out.

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11. INTERPRETATION OF RESULTS

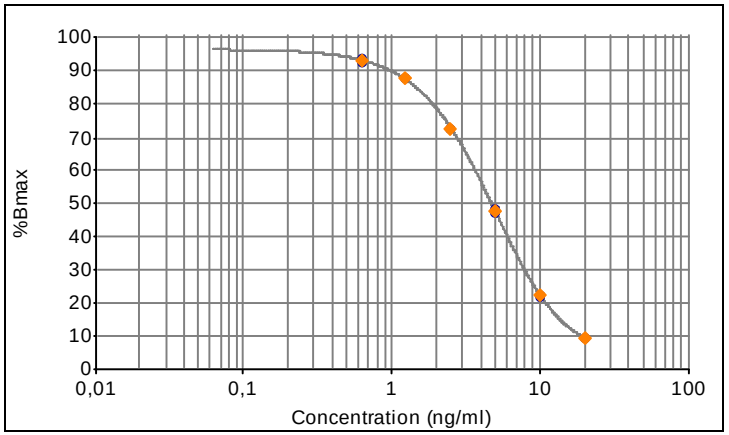
Subtract the mean optical density (O.D.) of the wells H1 and H2 (Blank) from the individual O.D. of the wells containing the standards and the samples.

The O.D. values of the 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

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

8.1 Milk samples
The bacitracin equivalents, as read from the standard curve, have to be multiplied by a factor 10 to obtain the bacitracin content in milk samples.

BRIEF INFORMATION

The Bacitracin ELISA is a competitive enzyme immunoassay for the screening of bacitracin in various matrices. The test is based on antibodies against Zinc bacitracin. 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 bacitracin from different matrices 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 BACITRACIN ELISA

The kit is based on a microtiter plate (12 strips, each 8 wells), precoated with specific antibodies (rabbit anti-bacitracin). Horseradish peroxidase labelled bacitracin (enzyme conjugate) and bacitracin standards or samples are added to the precoated wells followed by a single incubation step. Free bacitracin (in the standard solution or in the sample) and enzyme labelled bacitracin 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 bacitracin concentration in the sample.

3. SPECIFICITY AND SENSITIVITY

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

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	10 ng/ml
Egg	8.2	11 ng/ml
Tissue	8.2	9 ng/ml
Feed	8.3	60 ng/ml
Urine	8.4	23 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).

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).

2. Pipette 100 µl of the zero standard in duplicate (wells H1,H2, blank).
   Pipette 50 µl of the zero standard (Bmax) in duplicate (wells A1, A2).
   Pipette 50 µl of each of the Bacitracin standard solutions in duplicate (wells B1, 2 to G1, 2 i.e. 0.625, 1.25, 2.5, 5.0, 10.0 and 20 ng/ml).

3. Pipette 50 µl of each of the sample solution in duplicate into the remaining wells of the microtiter plate.

4. Pipette 50 µl conjugate solution to all wells, except wells H1 and H2.

5. Seal the microtiter plate and shake the plate for a few seconds on a microtiter plate shaker.

6. Incubate 1 hour at 20°C – 25°C in the dark.

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 for 30 minutes at 20°C – 25°C in the dark.

10 Pipette 100 µl of stop solution to each well.

11. Read the absorbance values immediately at 450 nm.

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 100 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 dilution buffer (no.9, chapter 5). Per 2 x 8 wells 800 µl is required. Store unused concentrated conjugate at 2°C to 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 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 (20°C to 25°C) and mix the content well before use. Avoid direct (sun) light.

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 done as follows:

Manual rinsing

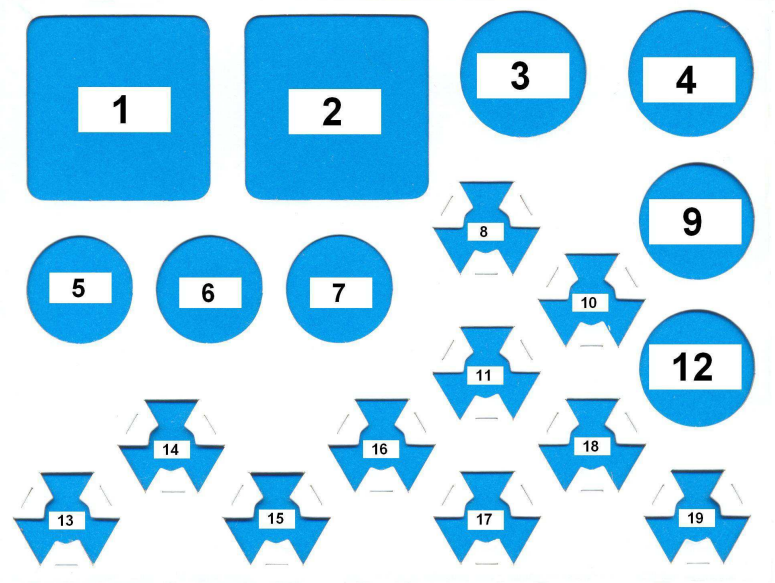
1. Empty the contents of each well by turning the microtiter plate upside down and remove residual liquid by striking the plate against a paper towel.
2. Fill all the wells to the rims (300 µl) with rinsing solution.
3. This rinsing cycle (1 and 2) should be carried out 3 times.
4. Turn the plate upside 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.

5. KIT CONTENTS

Manual

One sealed microtiter plate (12 strips, 8 wells each), coated with antibodies to bacitracin. 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. not in use
6. not in use
7. not in use
8. **Conjugate solution** (100x concentrated)
9. **Dilution buffer**
10. not in use
11. not in use
12. not in use
13. **Zero standard solution** (2ml, Ready-to-use)
14. **Standard solution 1** (1ml, ready-to-use) **0.625 ng/ml**
15. **Standard solution 2** (1ml, ready-to-use) **1.25 ng/ml**
16. **Standard solution 3** (1ml, ready-to-use) **2.5 ng/ml**
17. **Standard solution 4** (1ml, ready-to-use) **5 ng/ml**
18. **Standard solution 5** (1ml, ready-to-use) **10 ng/ml**
19. **Standard solution 6** (1ml, ready-to-use) **20 ng/ml**

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. SAFETY PRECAUTIONS

- Bacitracin is a toxic compound. Avoid contact with mouth and skin.
- 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 (5 minutes, 2000 x g, 4ºC).
- Remove the upper fat layer using a spatula.
- Dilute the defatted milk sample 10 times in sample dilution buffer (20 µl defatted milk plus 180 µl sample dilution buffer).
- Mix the diluted milk (vortex).
- An aliquot of 50 µl is used in the ELISA test.

8.2 Egg and tissue samples
- Homogenize 10 to 100 gram sample
- Weigh 1 gram of the homogenized sample and add 2 ml of 80% methanol in sample dilution buffer (1.6 ml 100% methanol plus 0.4 ml sample dilution buffer)
- Mix (15 minutes head-over-head)
- Centrifuge (10 minutes, 2000 x g)
- Dilute the supernatant 1:5 with sample dilution buffer (40 µl supernatant plus 160 µl sample dilution buffer).
- Vortex
- Use 50 µl of diluted sample in the ELISA test

8.3 Feed samples
- Homogenize 10 to 100 gram of feed samples
- Weigh 1 gram of the homogenized feed sample and add 2 ml of 80% methanol in sample dilution buffer (1.6 ml methanol in 0.4 ml sample dilution buffer)
- Mix (15 minutes head-over-head)
- Centrifuge (10 minutes, 2000 x g)
- Dilute the supernatant 1:20 with sample dilution buffer (10 µl supernatant with 190 µl sample dilution buffer)
- Vortex
- Use 50 µl of diluted sample in the ELISA test

8.4 Urine samples
- Urine samples can be analysed after a 25 times dilution step in sample dilution buffer (Paragraph 5, no. 1)

Dilution: Add to 20 µl urine 480 µl sample dilution buffer