LINCOMYCIN ELISA

5151LIN[4]04.20

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

EUROPROXIMA LINCOMYCIN ELISA

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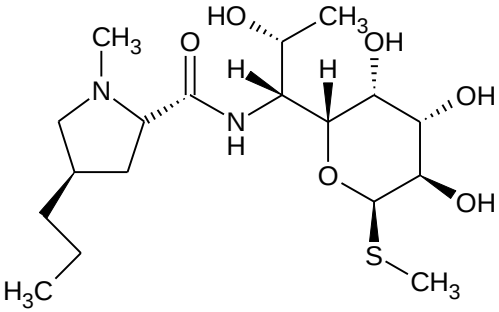
BRIEF INFORMATION

The Lincomycin ELISA is a competitive enzyme immunoassay. The test is based on antibodies directed against lincomycin. The ELISA kit contains a 96 well microtiter plate as well as all essential reagents including standards to perform the test. Methods for a fast and efficient extraction of lincomycin from different matrices are included in the kit manual.

1. INTRODUCTION

Lincomycin is a narrow spectrum antibiotic derived from *Streptomyces lincolnensis*. It belongs to the lincosamide group which also includes pirlimycin and clindamycin. Lincomycin is active mainly against gram-positive bacteria and cell wall-less bacteria including pathogenic species of *Streptococcus*, *Staphylococcus,* and *Mycoplasma*. It shows weak activity against most gram-negative bacteria. Lincomycin is also used to control American foulbrood disease in honey bees, caused by the spore-forming bacterium *Paenibacillus larvae* subsp. *larvae*.
Lincosamides exert their antibacterial action by inhibiting RNA-dependent protein synthesis by acting on the 50S subunit of the ribosome. Although they are chemically distinct from the macrolide antibiotics, lincosamides possess many pharmacologic similarities to them.
Lincomycin present in food products such as honey and milk may cause bacterial resistance and/or allergic reactions. For this reason, the EU has set maximum residue limits (MRLs) for lincomycin in different foods of animal origin: 50 µg/kg for fat/eggs, 100 µg/kg for muscle, 150 µg/kg for milk, 500 µg/kg for liver and 1500 µg/kg for kidney. Although in the EU no MRLs have been established for antibiotics in honey, some countries including Belgium, Hungary, Switzerland and the United Kingdom have set Action Limits, which vary from 10 to 50 µg/kg.



Chemical structure of lincomycin

2. PRINCIPLE OF THE LINCOMYCIN ELISA

The microtiter plate based lincomycin ELISA consists of one precoated plate (12 strips, 8 wells each). Antibody, horseradish peroxidase (HRP) labeled lincomycin and standard solution or sample are added to the wells. Free lincomycin from the samples or standards and lincomycin-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 lincomycin-HRP conjugate is visualized by the addition of a substrate/chromogen solution (H_2O_2/TMB). Bound lincomycin-HRP conjugate transforms the colourless chromogen into a coloured product.
The substrate reaction is stopped by the addition of sulphuric acid. The colour intensity is measured photometrically at 450 nm. The optical density is inversely proportional to the lincomycin concentration in the sample.

3. SPECIFICITY AND SENSITIVITY

The lincomycin ELISA utilizes antibodies raised against protein conjugated lincomycin. The reactivity pattern of the antibody is:

Cross-reactivity: Lincomycin 100%
Clindamycin <0.1%

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) and the detection capability (ccβ) are determined under optimal conditions. Cut-off criteria need critical consideration.

Matrix	Procedure	LOD (ppb)	ccβ (ppb)
Milk	8.1.1	45	75
Milk (sensitive)	8.1.2	0,6	1
Tissue	8.2	41	50
Liver	8.3	100	250
Honey	8.4	7	10
Egg	8.5	20	25

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).

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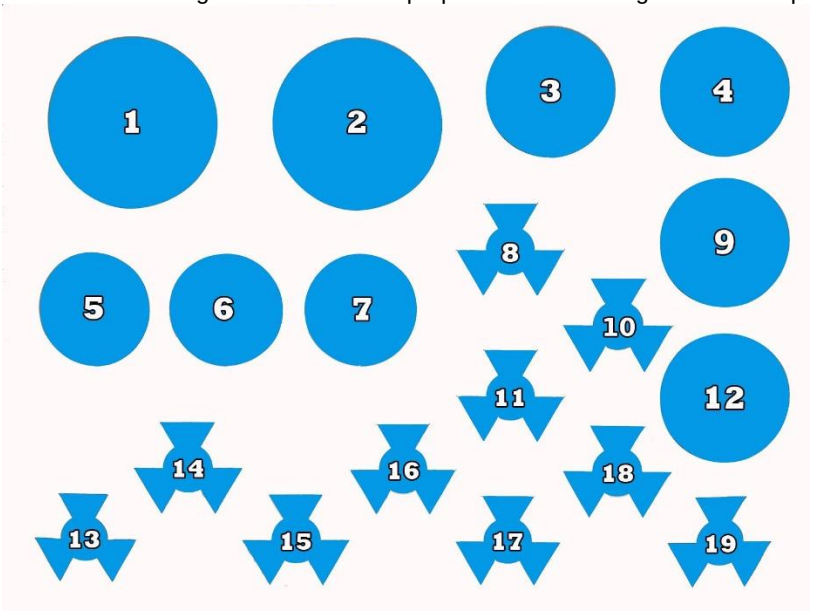
5. KIT CONTENTS

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. **Dilution buffer** (25 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. not in use
6. not in use
7. not in use
8. **Conjugate solution** (100 µl; 100x concentrated)
9. not in use
10. **Antibody solution** (100 µl; 100x concentrated)
11. not in use
12. not in use
13. **Zero standard solution** (2ml, ready-to-use)
14. **Standard solution 1** (1ml, ready-to-use) **0.0625 ng/ml**
15. **Standard solution 2** (1ml, ready-to-use) **0.125 ng/ml**
16. **Standard solution 3** (1ml, ready-to-use) **0.25 ng/ml**
17. **Standard solution 4** (1ml, ready-to-use) **0.5 ng/ml**
18. **Standard solution 5** (1ml, ready-to-use) **1.0 ng/ml**
19. **Standard solution 6** (1ml, ready-to-use) **2.0 ng/ml**

6. EQUIPMENT AND MATERIALS REQUIRED BUT NOT PROVIDED

- Scales and weighing vessels
- Gloves
- Homogeniser (vortex, mixer)
- 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
- Distilled water
- Methanol 100%

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

8.1.1 Milk
- Vortex the milk sample for 3 seconds
- Dilute 0.5 ml milk with 4.5 ml distilled water
- Vortex for 1 second
- Centrifuge (10 minutes, 2000 x g, 20°C to 25°C)
- Dilute 20 µl of the upper layer with 780 µl sample dilution buffer (see chapter 9)
- Vortex
- Use 50 µl in the ELISA.

8.1.2 Milk sensitive
- Vortex the milk sample for 3 seconds
- Dilute 250 µl milk with 250 µl sample dilution buffer (see chapter 9)
- Vortex for 3 seconds
- Use 50 µl in the ELISA.

8.2 Tissue
- Weigh 0.5 gram homogenized sample into a tube
- Add 4.5 ml distilled water
- Vortex, mix for 15 minutes head over head (rotor)
- Centrifuge (10 minutes, 2000 x g, 20°C to 25°C)
- Dilute 20 µl of the upper layer with 780 µl sample dilution buffer (see chapter 9)
- Use 50 µl in the ELISA

8.3 Liver
- Weigh 0.5 gram homogenized sample into a tube
- Add 4.5 ml distilled water
- Vortex, mix for 15 minutes head over head (rotor)
- Centrifuge (10 minutes, 2000 x g, 20°C to 25°C)
- Dilute 10 µl of the upper layer with 990 µl sample dilution buffer (see chapter 9)
- Vortex
- Use 50 µl in the ELISA.

8.4 Honey
- Weigh 1 gram homogenized sample into a tube
- Add 4 ml distilled water
- Vortex, mix for 15 minutes head over head (rotor)
- Centrifuge (10 minutes, 2000 x g, 20°C to 25°C)
- Dilute 25 µl of the upper layer with 475 µl sample dilution buffer (see chapter 9)
- Vortex
- Use 50 µl in the ELISA.

8.5 Egg
- Weigh 0.5 gram homogenized sample into a tube
- Add 4.5 ml distilled water
- Vortex, mix for 15 minutes head over head (rotor)
- Centrifuge (10 minutes, 2000 x g, 20°C to 25°C)

- Dilute 10 µl of the upper layer with 190 µl sample dilution buffer (see chapter 9)
- Vortex
- Use 50 µl in the ELISA.

9. PREPARATION OF REAGENTS

Before starting 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 fresh 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
This buffer is used for the dilution of conjugate, antibody and samples. The dilution buffer is 4x concentrated. Dilute the buffer 1:4 (10 ml buffer + 30 ml distilled water) before use. The concentrated buffer should be at room temperature (20ºC to 25ºC) and thoroughly mixed. Concentrated buffer can show precipitates, mix well before dilution.

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.

Conjugate
The conjugate is 100x concentrated. Spin down the conjugate in the vial by a short centrifugation step (1 minute, 1000 x g). Add 5 µl of the concentrated conjugate solution to 495 µl dilution buffer. Per 2 x 8 wells 400 µl is required. Store unused concentrated conjugate at 2°C to 8°C.

Antibody
The antibody is 100x concentrated. Spin down the antibody in the vial by a short centrifugation step (1 minute, 1000 x g). Add 5 µl of the concentrated antibody to 495 µl dilution buffer. Per 2 x 8 wells 400 µl of antibody solution is required. Store concentrated antibody immediately upon use at 2°C to 8°C.

Rinsing buffer
The rinsing buffer is delivered 20x 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.

10. ASSAY PROCEDURE

Rinsing protocol

Unbound components have to be removed efficiently between each incubation step in ELISAs. This is achieved by appropriate rinsing. Each rinsing procedure must be carried out with care to guarantee good inter- and intra-assay results.
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. Do not allow the wells dry out before the next reagent is dispensed.

Rinsing with automatic microtiter plate wash equipment

When using automatic plate wash equipment, make sure that all wells can be aspirated completely and 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 dilution buffer in duplicate (wells H1, H2, blank).
   Pipette 50 µl of dilution buffer (zero standard, Bmax) in duplicate (wells A1, A2).
   Pipette 50 µl of each of the standard 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 25 µl of conjugate (lincomycin-HRP) to all wells, except H1 and H2.

5. Pipette 25 µl of antibody solution to all wells except H1 and H2.

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

7. Incubate for 1 hour in the dark at 20ºC to 25ºC.

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

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

10. Incubate 30 minutes in the dark at 20ºC to 25ºC.

11. Add 100 µl of stop solution to each well.

12. Read the absorbance values immediately at 450 nm.

11. INTERPRETATION OF THE 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.

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

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 absorbance value of the standards is plotted on the Y-axis versus the concentration on the X-axis. The scale of the Y-axis is in logit and the X-axis is logarithmic.

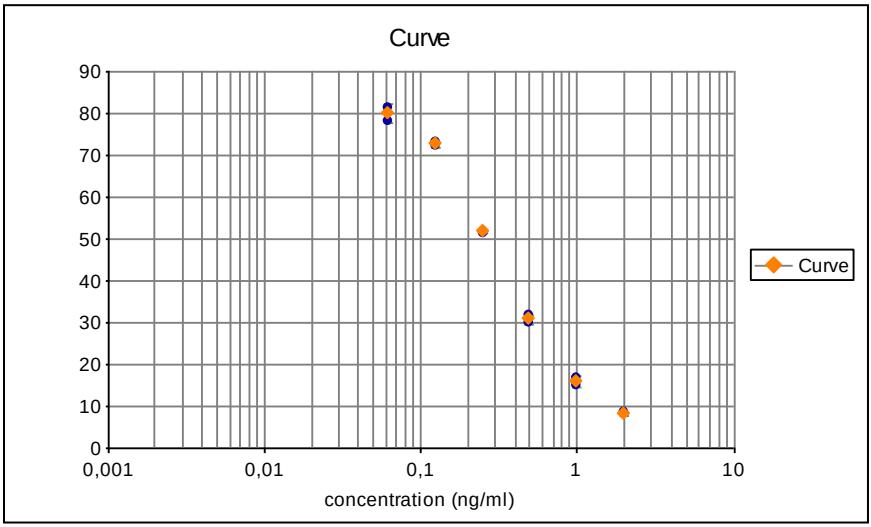


Figure 1: Example of a calibration curve

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

8.1.1 Milk samples
The lincomycin equivalents read from the calibration curve have to be multiplied by a factor 400 to obtain the lincomycin content in milk samples.

8.1.2 Milk samples (sensitive)
The lincomycin equivalents read from the calibration curve have to be multiplied by a factor 2 to obtain the lincomycin content in milk samples.

8.2 Tissue samples
The lincomycin equivalents read from the calibration curve have to be multiplied by a factor 400 to obtain the lincomycin content in tissue samples.

8.3 Liver samples
The lincomycin equivalents read from the calibration curve have to be multiplied by a factor 1000 to obtain the lincomycin content in liver samples.

8.4 Honey samples
The lincomycin equivalents read from the calibration curve have to be multiplied by a factor 100 to obtain the lincomycin content in honey samples.

8.5 Egg samples
The lincomycin equivalents read from the calibration curve have to be multiplied by a factor 200 to obtain the lincomycin content in egg samples.

12. LITERATURE

Feldlaufer M.F., Pettis J.S., Kochansky J-P., and Stiles G. Lincomycin hydrochloride for the control of American Foulbrood disease of honey bees. Apidologie 2001; **32**: 547-554.

Thompson T.S., Noot D.K., Calvert J., and Pernal S.F. Determination of lincomycin and tylosin residues in honey by liquid chromatography/tandem mass spectrometry. Rapid Commun. in Mass Spectrom. 2005; **19**: 309-316.

Commission Regulation (EU) No 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin. Off. J. European Union, **L15**, 1-72.

Bogdanov S. Contaminants of bee products. Apidologie 2006; **37**: 1-18.

13. ORDERING INFORMATION

For ordering the lincomycin ELISA kit, please use cat.code 5151LIN.

14. REVISION HISTROY

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

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