NEOMYCIN ELISA

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

EUROPROXIMA NEOMYCIN ELISA

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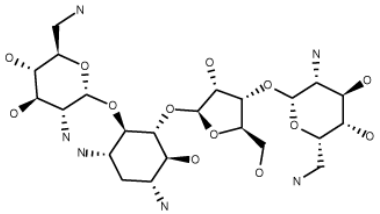
BRIEF INFORMATION

The neomycin ELISA is a competitive enzyme immunoassay for the screening of milk, milk powder, tissue, honey, serum/plasma and urine samples on the presence of this broad spectrum antibiotic. The test is based on antibodies directed against neomycin. 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 neomycin from different matrices are included in the kit manual.

1. INTRODUCTION



Chemical structure of Neomycin

Neomycin belongs to a group of carbohydrate containing antibiotics called aminoglycosides. All the aminoglycosides are potentially toxic compounds causing significant damage in vestibular and auditory functions in human as well as in animals. Nevertheless, they are used in practice because of their antibacterial and antifungal activities. These compounds have been found to be useful for the treatment of serious infections due to Gram negative micro-organisms. However, the range between therapeutic effectiveness and toxicity is narrow, therefore, dosage must be monitored. Aminoglycoside residues may occur in products of animal origin for several reasons such as deliberate feeding, inadvertent feeding to prevent infections in cows or to avoid outbreak of diseases of digestive and respiratory tracts of poultry.
Within the European Union, Maximum Residue Limits for aminoglycosides have been set (see Table I).

Table I: Maximum Residue Limits (µg/kg) for aminoglycosides.

Aminoglycoside	Kidney	Liver	Muscle	Milk	Fat	Egg
Streptomycin	1000	500	500	200	500	-
Dihydrostreptomycin	1000	500	500	200	500	-
Gentamicin	750	200	50	100	50	-
Neomycin	5000	500	500	1500	500	500

EuroProxima has also available a Gentamicin- and (Dihydro) Streptomycin ELISA.

2. PRINCIPLE OF THE NEOMYCIN ELISA

The kit is based on a microtiter plate (12 strips, each 8 wells), precoated with rabbit antibodies to mouse IgG. Monoclonal specific antibodies (mouse anti-neomycin), horseradish peroxidase labelled neomycin (enzyme conjugate) as well as neomycin standard solution or samples are pipetted into the precoated wells followed by a single incubation step. The specific antibodies are bound by the immobilised rabbit anti-mouse antibodies and simultaneously free neomycin (in the standard solution or in the sample) and enzyme labelled neomycin compete for the specific antibody binding sites (competitive enzyme immunoassay). After an incubation time of 1 hour, the non-bound (enzyme labelled) reagents are removed in a washing step.
The amount of neomycin enzyme conjugate is visualized by the addition of a chromogen substrate (tetramethylbenzidine, TMB). Bound enzyme conjugate 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 neomycin concentration in the sample.

3. SPECIFICITY AND SENSITIVITY

The neomycin ELISA utilizes a monoclonal antibody raised in mouse against protein conjugated Neomycin.

Cross- reactivity:	Neomycin	:	100%
	Gentamicin	:	< 0.1%
	Sisomycin	:	< 0.1%
	Kanamycin	:	< 0.1%
	Streptomycin	:	< 0.1%
	Dihydrostreptomycin	:	< 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	6.25*	10
Milk powder	8.1.2	6.25*	10
Tissue	8.2	31.25*	50
Honey	8.3	15.63*	25
Serum/plasma	8.4	6.25 *	6.25
Urine	8.5	8.42	10

* lowest standard multiplied by dilution factor

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:

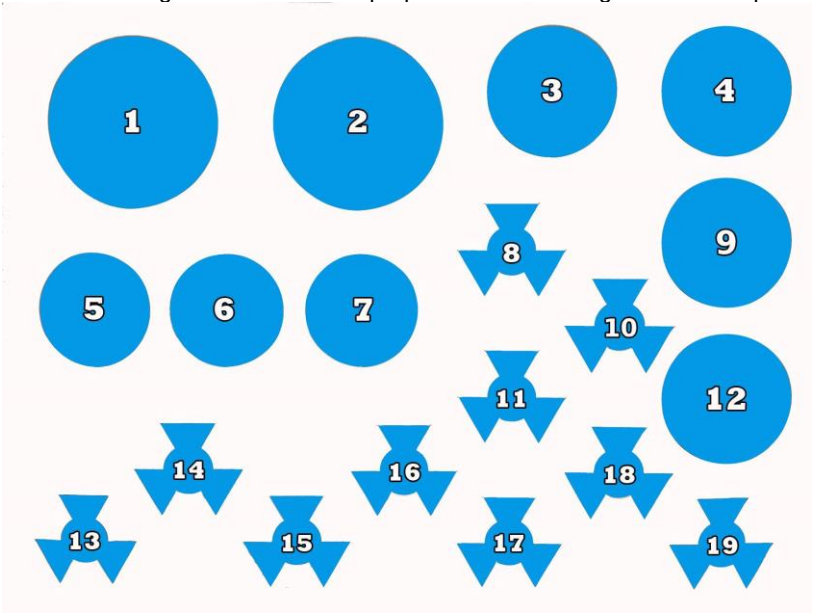
- A blue colouring of the chromogen solution before transferring it into the wells.
- A weak or no colour reaction in the zero standard wells (E450nm < 0.8).

5. KIT CONTENTS

Manual

One sealed microtiter plate (12 strips, 8 wells each), coated with antibodies to mouse IgG. Ready-to-use.

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



1. **Dilution buffer** (20 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. **Conjugate** (lyophilized, blue cap)
6. **Antibody** (lyophilized, yellow cap)
7. not in use
8. **Standard solution 1000 ng/ml** (1ml)
9. not in use
10. not in use
11. not in use
12. not in use
13. **Zero Standard** (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

- Scales and weighing vessels
- Gloves
- Fume hood
- Homogeniser (blender, Ultra Turrax, mixer)
- Centrifuge (for 10 – 15 ml test tubes, with cooling, 2000 x g)
- Vortex
- Automated microtiter plate washer or 8 channel micropipette 100 - 300 µl
- Magnetic stirrer
- Microtiter plate shaker
- Microtiter plate reader with 450 nm filter
- Siliconised glass test tubes or plastic tubes
- Micropipettes 20 - 200 µl, 100 - 1000 µl
- Multipipette with 2.5 ml combitips
- Aluminum foil or parafilm
- Distilled water
- Disodium hydrogen phosphate, Na_2HPO_4
- Potassium dihydrogen phosphate, KH_2PO_4
- Potassium chloride, KCl
- Sodium chloride, NaCl
- Tween 80

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 PREPARATIONS

8.1 Milk samples
- Dilute and homogenise the milk sample 10 times in sample dilution buffer (see chapter 9), e.g. 50 µl of milk added to 450 µl of sample dilution buffer
- Pipette 50 µl of this solution in the ELISA plate.

8.1.2 Milk powder samples
- Reconstitute 12 g of the milk powder in 88 ml of distilled water or reconstitute the milk powder according to manufacturers' instruction.
- Mix well till a homogeneous solution
- Dilute the reconstituted milk powder 10 times in sample dilution buffer (see Chapter 9) e.g. 50 µl of reconstituted milk powder added to 450 µl of sample dilution buffer.
- Pipette 50 µl of this solution in the ELISA plate.

8.2 Tissue samples
- Weigh 5 gram finely cut subsequently homogenized tissue in a plastic tube
- Add 20 ml sample dilution buffer (see Chapter 9)
- Homogenise (for instance using an Ultra Turrax or head over head mixer) for 30 minutes
- Centrifuge a part of the mixture 10 minutes at 4000 x g at 4ºC
- Remove the upper fat layer
- Pipette 50 µl supernatant into a plastic tube, add 450 µl sample dilution buffer, vortex
- Pipette 50 µl of this solution in the ELISA plate

8.3 Honey samples
- Weigh 1 g of homogenized honey in a plastic tube.
- Add 4 ml of sample dilution buffer (see Chapter 9) and mix well using a vortex
- Wait for a minute to obtain a separation between the solid part and the liquid part in the sample.
- Pipette 1 ml of the clear upper liquid into a clean tube and add 4 ml of sample dilution buffer
- Mix well using a vortex
- Pipette 50 µl of this solution in the ELISA plate

8.4 Serum / plasma samples
- Dilute serum samples 10 times in sample dilution buffer (see Chapter 9).
- E.g. pipette 50 µl of serum sample into a clean siliconised glass tube or plastic vial.
- Add 450 µl of sample dilution buffer.
- Mix well using a vortex
- Pipette 50 µl of this solution in the ELISA plate

8.5 Urine samples
- Dilute urine samples 10 times in sample dilution buffer (see Chapter 9)
- Check pH 7.4 $\pm$ 0.4.
- Pipette 50 µl of this solution in the ELISA plate

8.6 Egg samples
- Weigh 0.5 gram whole (homogenized) egg in a plastic tube.
- Add 4.5 ml sample dilution buffer (see Chapter 9) and vortex
- Homogenise (for instance using an Ultra Turrax or head over head mixer) for 30 minutes
- Centrifuge the mixture 10 minutes at 2000 x g at 4ºC
- Pipette 50 µl of the supernatant in the ELISA plate

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.

Rinsing buffer
The rinsing buffer is delivered 20 times concentrated. Prepare dilutions freshly before use. Per strip 20 ml of diluted rinsing buffer is used (1 ml concentrated rinsing buffer + 19 ml distilled water).

Substrate solution
The substrate solution (ready-to-use) precipitates at 4ºC. Take care that this vial is at 20°C to 25°C (keep in the dark) and mix the content before pipetting into the wells.

Standard solutions 1000 ng/ml
To prepare standards in the appropriate matrix or to prepare spikes use the standard solution containing 1000 ng neomycin per ml. Dilute the standard solution in the appropriate matrix to make a dilution range of 20, 10, 5, 2.5, 1.25, 0.625 ng/ml.

Conjugate solution
Reconstitute the vial of lyophilized conjugate (Neomycin-HRP) with 4 ml of dilution buffer, mix thoroughly and keep in the dark until use.

Antibody solution
Reconstitute the vial of lyophilized antibodies with 4 ml of dilution buffer, mix thoroughly and keep in the dark until use.

Sample dilution buffer
Dissolve in 1 L distilled water
1.15 g Na_2HPO_4
0.2 g KH_2PO_4
0.2 g KCl
30 g NaCl
0.5 ml Tween 80
Adjust pH to 7.4

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.

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 zero standard in duplicate (wells H1, H2; blank).
   Pipette 50 µl of zero standard in duplicate (wells A1, A2; maximal O.D.).
   Pipette 50 µl of each standard dilution in duplicate (well B1,2 to G1,2 i.e. 0.625, 1.25, 2.5, 5, 10, 20 ng neomycin/ml).
   Pipette 50 µl of each sample solution in duplicate into the remaining wells of the microtiter plate.

3. Pipette 25 µl of conjugate (neomycin-HRP) into all wells, except wells H1 and H2.

4. Pipette 25 µl of antibody solution into all wells, except wells H1 and H2.

5. Seal the microtiter plate and shake the plate for 1 minute on a microtiter plate shaker.

6. Incubate for 1 hour in the dark at 4ºC.

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 at 20ºC - 25ºC.

10. Pipette 100 µl of stop solution into 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 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 (percentage 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.

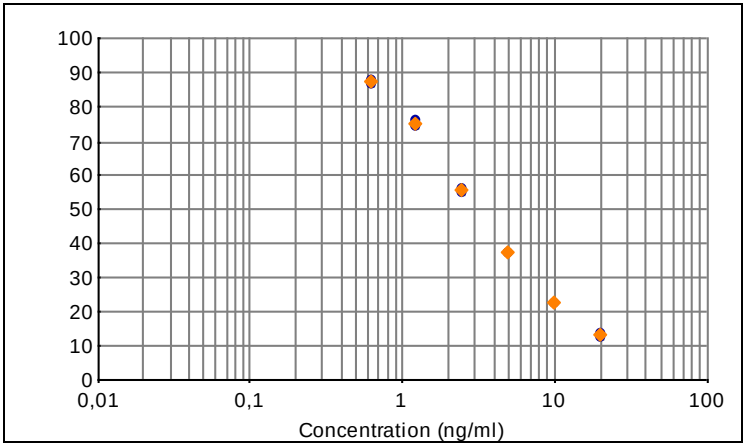


Figure 1: Example of a calibration curve

8.1 Milk samples:
The neomycin equivalents read from the calibration curve have to be multiplied by a factor 10.

8.1.2 Milk powder samples
The neomycin equivalents read from the calibration curve have to be multiplied by a factor 10.

8.2 Tissue samples:
The neomycin equivalents read from the calibration curve. have to be multiplied by a factor 50

8.3 Honey samples
The neomycin equivalents read from the calibration curve have to be multiplied by a factor 25.

8.4 Serum / plasma samples
The neomycin equivalents read from the calibration curve have to be multiplied by a factor 10.

8.5 Urine samples
The neomycin equivalents read from the calibration curve have to be multiplied by a factor 10.

8.6 Egg samples
The neomycin equivalents read from the standard curve have to be multiplied by a factor 10.

12. LITERATURE

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Standefer J.C. and Saunder G.C. Enzymeimmunoassay for Gentamicin, Clin. Chem., **24**, 1903, 1978.

Analysis of Antibiotic Drug Residues in Food Products of Animal Origin., Ed. Vipin K. Agarwal U.S.A. 1992.

Commission Regulation 37/2010/EU. Official J. of the European Union, L15 (2010) 1-72.

13. ORDERING INFORMATION

For ordering the Neomycin ELISA kit, please use cat. code 5111NEO.

14. REVISION HISTORY

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

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