NEOMYCIN ELISA

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A competitive enzyme immunoassay for screening and quantitative analysis of neomycin in milk, milk powder, tissue, honey, serum/plasma and urine samples

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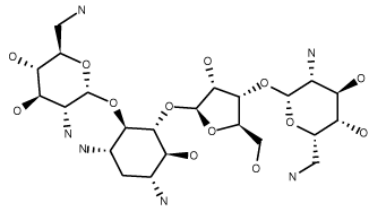
BRIEF INFORMATION

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 [1]. All the aminoglycosides are potentially toxic compounds [2,3] 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) [4].

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

Matrix	Procedure	LOD ppb	CCβ
Milk	8.1	6.25*	10 ng/ml
Milk powder	8.1.2	6.25*	10 ng/g
Tissue	8.2	31.25*	50 ng/g
Honey	8.3	15.63*	25 ng/g
Serum/plasma	8.4	6.25 *	6.25 ng/ml
Urine	8.5	8.42	10 ng/ml

* lowest standard multiplied by dilution factor

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

Remark: "positive" samples have to be confirmed by alternative (e.g. chromatographic) methods.

12. LITERATURE

1. Berdy J., Aszalos A., Bostian M and McNitt K.L. Handbook of Antibiotic Compounds, Vol. 1, CRC Press Inc. Boca Raton, Florida U.S.A. 1980.

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

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

4. 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. LAST MUTATIONS

Chapter 8 and Chapter 11: instructions for Egg samples added.

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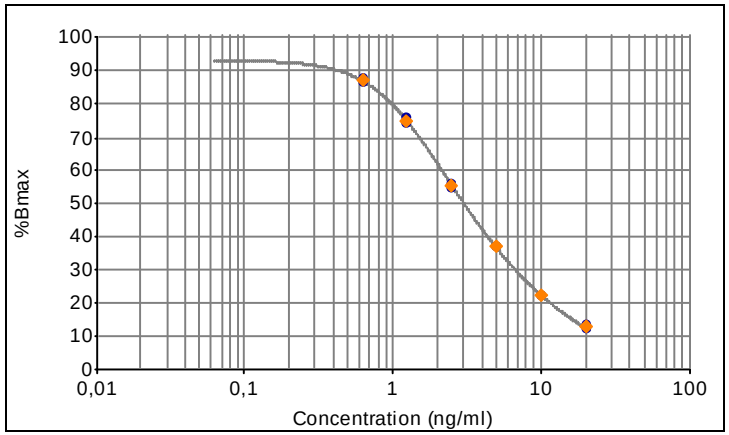


Figure 1: Example of a calibration curve

8.1 Milk samples:
The amount of neomycin in the milk samples is expressed as neomycin equivalent (ng/ml). The neomycin equivalents in the milk (ng/ml) corresponding to the % maximal absorbance of each sample can be read from the calibration curve. These calculated neomycin equivalents have to be multiplied by 10 to obtain the neomycin equivalents (ng/ml) in the undiluted milk sample.

8.1.2 Milk powder samples
The amount of neomycin in the milk samples is expressed as neomycin equivalent (ng/ml). The neomycin equivalents in the milk (ng/ml) corresponding to the % maximal absorbance of each sample can be read from the calibration curve. These calculated neomycin equivalents have to be multiplied by 10 to obtain the neomycin equivalents (ng/ml) in the undiluted milk sample.

8.2 Tissue samples:
The neomycin equivalents in the tissue extract corresponding to the % maximal absorbance of each sample can be read from the calibration curve. These equivalents have to be multiplied by 50 to obtain the neomycin equivalents (ng/g) in the tissue samples.

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

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

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

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.

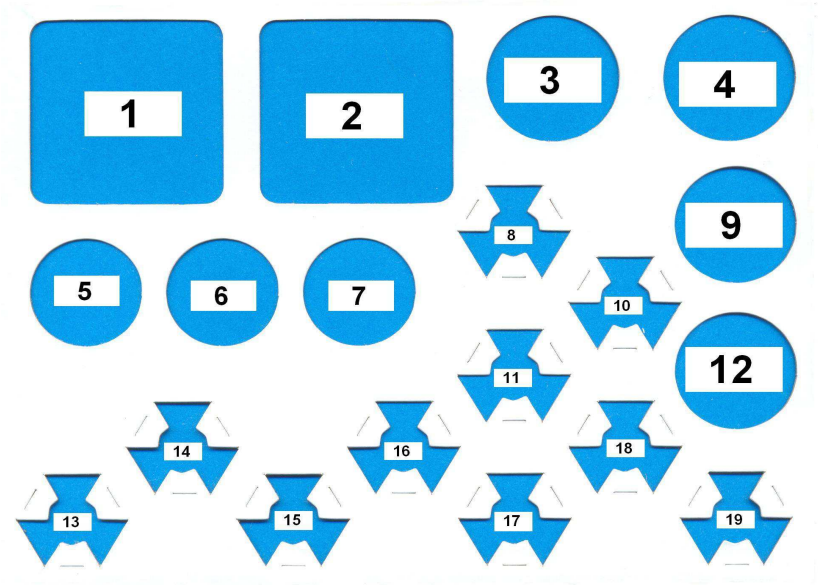
Degeneration of the reagents may have occurred when the following phenomena are observed:
- A blue colouring of the chromogen solution before transferring it into the wells.
- A weak or absent colour reaction of the maximum binding (zero standard) (E450nm < 0.8).

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. 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.

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

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.

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 (Sample preparation) and prepare reagents according to Chapter 9 (Preparation of reagents).

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. 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

7. PRECAUTIONS

- Neomycin is a toxic compound. Avoid contact with mouth and skin. Be aware that neomycin 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 PREPARATIONS

8.1 Milk samples
- Dilute and homogenise the milk sample 10 times in **sample dilution buffer (SDB), e.g. 50 µl of milk added to 450 µl of **SDB.
- 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 **SDB e.g. 50 µl of reconstituted milk powder added to 450 µl of **SDB
- Pipette 50 µl of this solution in the microtiter plate.

8.2 Tissue samples
- Weigh 5 gram finely cut subsequently homogenized tissue in a plastic tube
- Add 20 ml **SDB
- 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 **SDB, vortex
- Use 50 µl of this solution in the ELISA

8.3 Honey samples
- Weigh 1 g of homogenized honey in a plastic tube.
- Add 4 ml of **SDB 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 **SDB
- Mix well using a vortex
- Use 50 µl of the dilution in the ELISA.

8.4 Serum / plasma samples
- Dilute serum samples 10 times in **SDB.
- E.g. pipette 50 µl of serum sample into a clean siliconised glass tube or plastic vial.
- Add 450 µl of **SDB.
- Mix well using a vortex
- Use 50 µl of this 10 times diluted serum (in duplicate) in the ELISA.

8.5 Urine samples
- Dilute urine samples 10 times in **SDB.
- Check pH 7.4 $\pm$ 0.4.
- Use 50 µl of the dilution in the ELISA.

** SDB: 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 (pH 7.4)

8.6 Egg samples
- Weigh 0.5 gram whole (homogenized) egg in a plastic tube.
- Add 4.5 ml *SDB 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
- Use 50 µl supernatant in the ELISA

* SDB: 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 (pH 7.4)

9. PREPARATION OF REAGENTS

Ready-to-use standards are prepared in dilution buffer. When an alternative sample matrix is used standards or spikes have to be prepared in the sample matrix from the enclosed 1000 ng/ml standard solution.

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 40 ml of diluted rinsing buffer is used (2 ml concentrated rinsing buffer + 38 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. Also the zero standard should be of the same matrix.

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.