GENTAMICIN ELISA

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

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8.6 Egg samples
Multiply the calculated gentamicin equivalents by 5 to obtain the gentamicin equivalents (ng/ml) in egg samples.

8.7 Urine samples:
Multiply the calculated gentamicin equivalents by 10 to obtain the gentamicin equivalents (ng/ml) in undiluted urine samples.

8.8 Feed samples:
Multiply the calculated gentamicin equivalents by 50 to obtain the gentamicin equivalents (ng/g) in feed samples

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. Enzyme immunoassay for Gentamicin, Clin. Chem.(1978), 24, 1903.
3. Analysis of Antibiotic Drug Residues in Food Products of Animal Origin., Ed. Vipin K. Agarwal U.S.A. 1992.
4. Haasnoot W., Stouten P., Cazemier G., Lommen A., Nouws J.F.M. and Keukens H.J. Immunochemical detection of Aminoglycosides in milk, and kidney. The Analyst, 1999, 124, 301-305.

13. ORDERING INFORMATION

For ordering the Gentamicin ELISA kit, please use cat. code 5111GEN.

14. LAST MUTATIONS

Standard solution 1000 ng/ml.
Pipette schedule of the standard curve is adapted.

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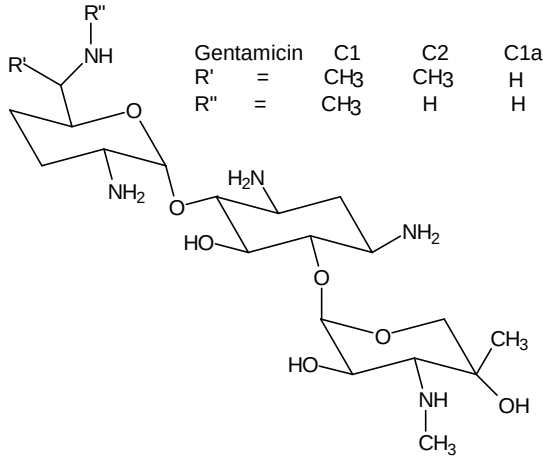
BRIEF INFORMATION

The Gentamicin ELISA is a competitive enzyme immunoassay for measurement of the concentration of gentamicin in various samples. With this ELISA kit 96 analyses can be performed. Samples and standards are measured in duplicate which means that a total 40 samples can be analyzed.
The ELISA kit contains all reagents to perform the assay. Reagents for sample preparation are not included in the kit.

1. INTRODUCTION



Gentamicin	C1	C2	C1a
R' =	CH_3	CH_3	H
R" =	CH_3	H	H

Gentamicin 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, provisional Maximum Residue Limits for aminoglycosides have been fixed (see Table I).

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

Aminoglycosides	Kidney	Liver	Muscle	Milk	Fat	Eggs
Streptomycin	1.0	0.5	0.5	0.2	0.5	-
Dihydrostreptomycin	1.0	0.5	0.5	0.2	0.5	-
Gentamicin	0.75	0.2	0.05	0.1	0.05	-
Neomycin	5.0	0.5	0.5	1.5	0.5	0.5

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

2. PRINCIPLE OF THE GENTAMICIN-ELISA

The microtiter plate based gentamicin ELISA consists of one precoated plate (12 strips, 8 wells each). Antibody, horseradish peroxidase (-HRP) labelled gentamicin and standard solution or sample are added to the wells. Free gentamicin from the samples or standards and gentamicin-HRP conjugate compete for the specific antibody binding sites (competitive enzyme immunoassay).
After an incubation time of 1 hour, the non-bound reagents are removed in a washing step. The amount of bound gentamicin-HRP conjugate is visualized by the addition of a substrate/chromogen solution (H_2O_2/TMB). Bound gentamicin-HRP conjugate transforms the colourless 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. The optical density is inversely proportional to the gentamicin concentration in the sample.

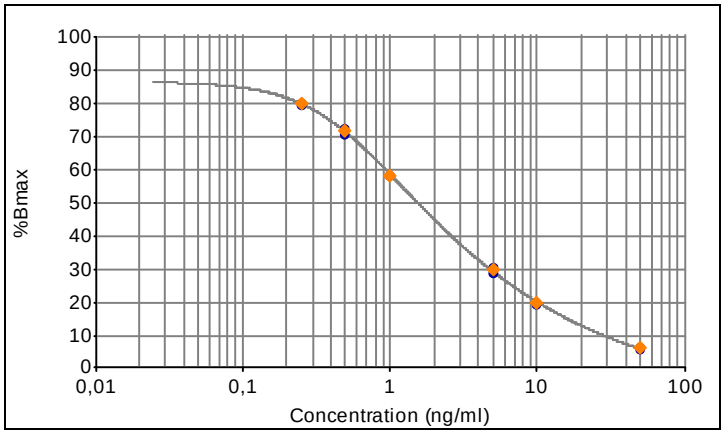


Figure 1: Example of a calibration curve

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

8.2 Tissue samples
The gentamicin 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 gentamicin equivalents (ng/g) in the tissue samples.

8.3 Fat samples
Multiply the calculated gentamicin equivalents by 50 to obtain the gentamicin equivalents (ng/g) in fat samples

8.4 Serum samples
Multiply the calculated gentamicin equivalents by 10 to obtain the gentamicin equivalents (ng/ml) in undiluted serum samples.

8.5.1Honey samples
Procedure I (dilution in buffer)
Multiply the calculated gentamicin equivalents by 25 to obtain the gentamicin equivalents (ng/g) in honey samples.

8.5.2 Procedure II (alternative method)
Multiply the calculated gentamicin equivalents by 10 to obtain the gentamicin equivalents (ng/g) in honey samples.

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 room temperature (20ºC to 25ºC).

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

12. 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 value of absorption (logit) calculation of the standards are plotted on Y-axis versus the analyte equivalent concentration on a logarithmic X-axis.

3. SPECIFICITY AND SENSITIVITY

The Gentamicin ELISA utilizes antibodies raised in rabbits against protein conjugated gentamicin. The reactivity pattern of the antibody is:

Cross- reactivity:	Gentamicin	100%
	Sisomycin	25%
	Neomycin	< 0.1%
	Kanamycin	< 0.1%
	Tobramycin	< 0.1%
	Lincomycin	< 0.1%

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

Matrix	Procedure	LOD (ppb)
Milk	8.1	2
Tissue	8.2	10
Serum	8.4	2
Honey	8.5.1	5
Honey	8.5.2	2.5
Egg	8.6	1
Urine	8.7	4
Feed	8.8	10

4. HANDLING AND STORAGE

- Store the kit at + 2ºC to + 8ºC in a dark place. For repeated use store kit components as specified under chapter 9.
- After the expiry date (see kit label) has passed, quality claims are not accepted.
- Before opening the sealed plate, the plate should be at ambient temperature in order to avoid condensation in the ELISA.
- Dilute the kit components immediately before use, but after the components are at ambient temperature.
- The substrate chromogen solution can be stored in a refrigerator (+2ºC to + 8ºC) until the expiry date stated on the label.
- Any direct action of light on the substrate chromogen solution should be avoided.

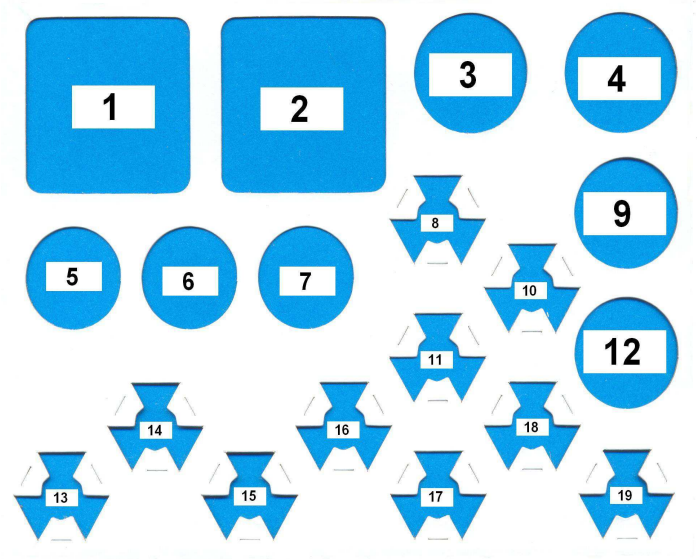
Degeneration of the reagents may have occurred when the following phenomena are observed:
- A blue colouring of the substrate solution before transferring it into the wells,
- A weak or absent colour reaction of the zero standard (Bmax, E450nm < 0.8).

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** (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) **0.25 ng/ml**
15. **Standard solution 2** (1ml, ready-to-use) **0.5 ng/ml**
16. **Standard solution 3** (1ml, ready-to-use) **1 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) **50 ng/ml**

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 washing 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 treatment) 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).
   Pipette 50 µl of each of the standard dilutions in duplicate (wells B1,2 to G1,2 i.e. 50,10, 5, 1, 0.5 and 0.25 ng gentamicin/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 (gentamicin-HRP) to all wells, except H1 and H2.

5. Add 25 µl of antibody solution into all wells, except wells 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 in a refrigerator (2ºC to 8ºC).

*** Extraction buffer: Dissolve in 1 L distilled water 10.1 g (50mM) 1-Heptanesulfonic acid (Sigma H2766) and 11.4 g (30mM) $Na_3PO_4.12H_2O$. Adjust to pH 2.0 with H_3PO_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 beginning 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 8 ºC. Prepare reagents fresh before use. For prolonged storage see chapter 4 (Handling and Storage).

Microtiter plate
Return unused strip into resealable bag with desiccant and store at 2ºC to 8ºC 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 room temperature (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 gentamicin per ml. Dilute the standard solution in the appropriate matrix to make a dilution range of 50, 10, 5, 1, 0.5, 0.25 ng/ml. Also the zero standard should be of the same matrix.

Conjugate solution
Reconstitute the vial of lyophilised conjugate (gentamicin-HRP) with 4 ml of dilution buffer, mix thoroughly and keep in the dark until use. Store in the dark at 2ºC to 8ºC for one week maximally.
For prolonged storage aliquot and freeze at -20ºC.

Antibody solution
Reconstitute the vial of lyophilised antibodies with 4 ml of dilution buffer, mix thoroughly and keep in the dark until use. Store the vial immediately after use in the dark at 2ºC to 8ºC for one week maximally.
For prolonged storage aliquot and freeze at -20ºC.

6. EQUIPMENT AND MATERIALS REQUIRED BUT NOT PROVIDED

- Scales and weighing vessels
- Gloves
- Fume hood
- Homogeniser (blender, Ultra Turrax, mixer)
- Centrifuge (2000 x g)
- Vortex
- Automated microplate washer or 8 channel micropipette 100 - 300 µl
- 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
- Methanol 100%

7. SAFETY PRECAUTIONS

- Gentamicin is a toxic compound. Avoid contact with mouth and skin. Be aware that gentamicin 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; take care when handling the substrate.
- 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, which crystallises 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 TREATMENT

8.1 Milk samples
- Defat milk by centrifugation for 10 minutes at 4ºC and 2000 x g.
- Eliminate the upper fat layer.
- Dilute and homogenize the defatted milk sample 10 times in SDB*
- Check pH 7.4 ± 0.4.
- Pipette 50 µl of this solution into 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 Fat samples
- Weigh 1 g of homogenized (melted) fat in a plastic tube.
- Add 10 ml of SDB* and heat in a water bath at 70ºC for 30 minutes.
- Centrifuge 15 minutes, 2000 x g, at 4ºC.
- Remove the upper fat layer and pipette 1 ml of sample extract in a plastic tube.
- Add 4 ml of SDB*, mix well.
- Pipette 50 µl of this diluted solution (containing 0.02 g of fat/ml) into the microtiter plate.

8.4 Serum 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 Honey samples

8.5.1 Procedure I (dilution in buffer)
- 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.

* 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).

8.5.2 Procedure II (alternative method)
- Add 9 ml of extraction buffer*** to 1 g of honey.
- Mix for 10 minutes at room temperature.
- Centrifuge solution for 10 minutes at 2000 x g.
- Condition Sep-pak C18 (Waters number 051910) cartridges with 10 ml of distilled water.
- Further condition with 5 ml of methanol and with 10 ml of distilled water.
- Transfer 5 ml of clear sample supernatant onto an activated column.
- Wash the column with 5 ml of distilled water
- Dry the column under vacuum.
- Elute gentamicin with 1 ml of methanol.
- Evaporate the eluate to dryness under a mild stream of nitrogen at 40ºC.
- Dissolve the dry extract in 5 ml of SDB* (0,1 g of sample/ml SDB), i.e. a dilution factor of 10.
- Use 50 µl of the dilution in the ELISA.

8.6 Egg samples
- Homogenise an egg, both egg-protein and yolk.
- Pipette 1 ml of the homogenised sample into a clean tube.
- Add 4 ml of SDB*
- Mix well using a vortex.
- Use 50 µl of the mixture in the ELISA.

8.7 Urine samples
- Dilute urine samples 10 times in SDB*.
- Check pH 7.4 ± 0.4.
- Use 50 µl of the dilution in the ELISA.

8.8 Feed samples
- Weigh 1 g finely cut and subsequently homogenised feed sample in the plastic tube.
- Add 5 ml of a trichloroacetic acid solution (3%)
- Homogenise (for instance using an Ultra Turrax) for 1 minute.
- Mix head over head for 30 minutes.
- Centrifuge 10 minutes at 2000 x g and 4ºC.
- Remove the upper fat layer eventually present.
- Pipette 100 µl of the supernatant filtrate into a plastic tube.
- Add 900 µl of SDB* and mix.
- Adjust pH to 7.4 ± 0.4.
- Pipette 50 µl of this solution (containing 0.02 g of feed/ml) into the microtiter plate.

* 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)

** Extraction-kit: the plastic tube, stopper and matching filter tube (40 of each) can be ordered separately (For ordering the extraction-kit, please use cat. code 8015Tubes40V).