STREPTOMYCIN ELISA

A microtiter plate based competitive enzyme immunoassay for the screening and quantitative analysis of Streptomycin and Dihydrostreptomycin in milk, tissue, fat, serum, honey, royal jelly, egg and urine samples

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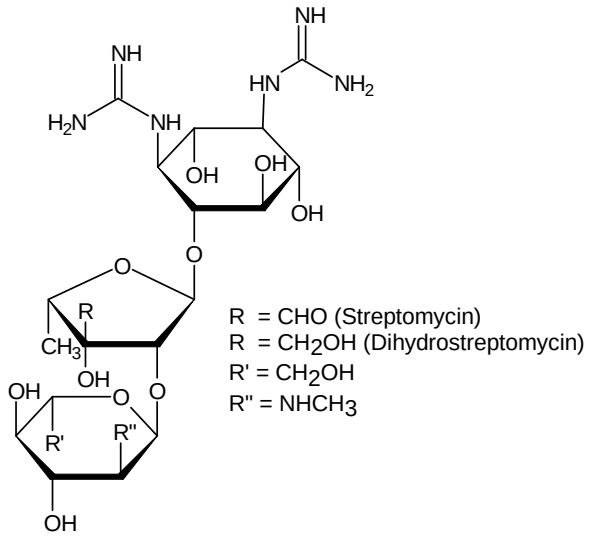
BRIEF INFORMATION

The Streptomycin ELISA is a competitive enzyme immunoassay for the screening and quantitative analysis of streptomycin in milk, tissue, fat, serum, honey, royal jelly, egg and urine samples. The test is based on a polyclonal antibody raised in rabbits against protein bound streptomycin.
With this ELISA kit 96 analyses can be performed. Samples and standards are measured in duplicate which means that in total 40 samples can be analysed. The kit contains all reagents required, including standards, to perform the test. Materials and chemicals necessary for extraction of streptomycin from sample material are not included in the test kit.

1. INTRODUCTION



Chemical structure of Streptomycin

Streptomycin and Dihydrostreptomycin belong 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 delibrate 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.

Aminoglycoside	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 a Gentamicin-ELISA.

2. PRINCIPLE OF THE STREPTOMYCIN-ELISA

The microtiter based ELISA kit consists of 12 strips, each 8 wells, precoated with sheep antibodies to rabbit IgG. A specific antibody (rabbit anti-Streptomycin), horseradish peroxidase labelled Streptomycin (enzyme conjugate) as well as Streptomycin standards or samples are pipetted into the precoated wells followed by a single incubation step. The specific antibodies are bound by the immobilised antibodies and simultaneously free Streptomycin (present in the standard solution or sample) and enzyme labelled Streptomycin compete for the Streptomycin 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 Streptomycin enzyme conjugate bound to the specific antibody is visualised by the addition of chromogen / substrate (tetramethylbenzidine, TMB). During the incubation the colourless chromogen is converted by the enzyme into a blue coloured reaction product. This blue colour is inversely proportional to the amount of bound Streptomycin. The more Streptomycin is present in the standard solution or sample, the less colour is developed. The substrate reaction is stopped by the addition of sulphuric acid. In the acetic environment the blue colour changes into a yellow colour. The colour intensity is measured photometrically at 450 nm.

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. (1978), 24, 903, 1987.
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 Streptomycin ELISA kit, please use cat. code 5111STREP.

14. LAST MUTATIONS

LOD tissue changed to 10 ppb.
8.2 Tissue samples: add 450 ml SDB changed to 450 µl SDB

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E-mail: info@europroxima.com

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

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

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

Honey samples (SPE solid phase extraction)
Multiply the calculated Streptomycin equivalents by 20 to obtain the Streptomycin equivalents in ng/g honey and royal jelly samples.

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

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

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

3. SPECIFICITY AND SENSITIVITY

The Streptomycin-ELISA utilizes a polyclonal antiserum raised in rabbits against protein conjugated Streptomycin.

Cross- reactivity:	Streptomycin	:	100	%
	Dihydrostreptomycin	:	100	%
	Neomycin	:	< 0.1	%
	Kanamycin	:	< 0.1	%
	Tobramycin	:	< 0.1	%
	Amikacin	:	< 0.1	%
	Gentamicin	:	< 0.1	%
	Sisomycin	:	< 0.1	%

The limit of detection (LOD) is calculated as: Xn ± 3SD. For extraction procedures the LOD is calculated reflecting the respective concentration steps. The LOD is determined under optimal conditions. Cut-off values need critical consideration.

Matrix	Procedure	LOD	
Milk	Defatting and dilution	4	ppb
Tissue	Buffer extraction	10	ppb
Serum	Dilution in buffer	2	ppb
Urine	Dilution in buffer	4	ppb
Honey	Dilution in buffer	6	ppb
Honey	Solid phase extraction	5	ppb
Royal Jelly	Solid phase extraction	5	ppb
Egg	Dilution in buffer	2	ppb

4. HANDLING AND STORAGE

- Store the kit at + 2ºC to + 8ºC in a dark place.
- Be aware: Aminoglycosides, inclusive Streptomycin, adsorb to glass. Always use siliconised glass tubes or plastic ones.
- After the expiry date (see kit label) has passed, it is no longer possible to accept any further quality guarantee.
- In order to avoid condensation in the ELISA plate, allow the sealed plate to equilibrate to room temperature before opening it.
- Reconstitute or dilute the kit components immediately before use, but after the components are at ambient temperature.

- After the lyophilised enzyme conjugate (Streptomycin-HRP) and the lyophilised antibodies have been reconstituted, the reconstituted components can be stored in a refrigerator for maximally one week (stored at + 2°C to + 8°C in the dark). For prolonged storage aliquot the reconstituted components and store at –20°C.

- The substrate and standard solutions can be stored in a refrigerator (+2°C to +8°C) until the expiration date stated on the label.

- Any direct action of light on the substrate / chromogen solution should be avoided.

If the following phenomena are observed, this may indicate a degradation of the reagents:

- A blue colouring of the substrate / chromogen solution before putting it into the wells,

- A weak or absent colour reaction of the maximum binding (zero standard) (E 450 nm < 0.8).

11. INTERPRETATION OF RESULTS

Subtract the mean optical density (O.D.) value of the blank wells A1 and A2 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 (wells B1 and B2) and multiplied by 100. The zero standard is thus made equal to 100% (maximal absorbance) and the other O.D. values are quoted in percentages of the maximal absorbance.

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

Calibration curve:

The values (% maximal absorbance) calculated for the standards are plotted (on the Y-axis) versus the Streptomycin equivalent concentration (ng/ml) on a logarithmic X-axis.

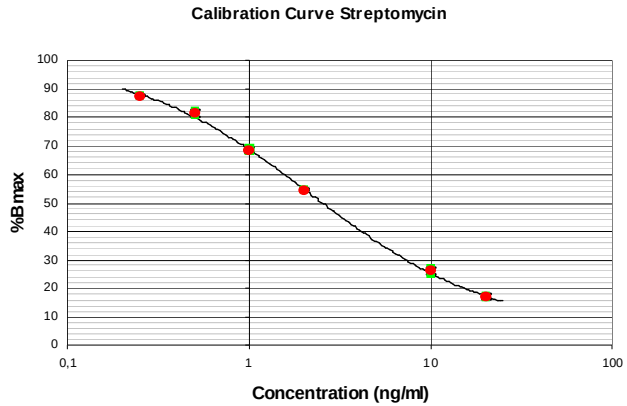


Figure 1: Example of a calibration curve

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

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

10. ASSAY PROCEDURE

Rinsing protocol

In ELISA's, between each immunological incubation step, un-bound 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 followed by a firm short vertical movement.
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 in 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

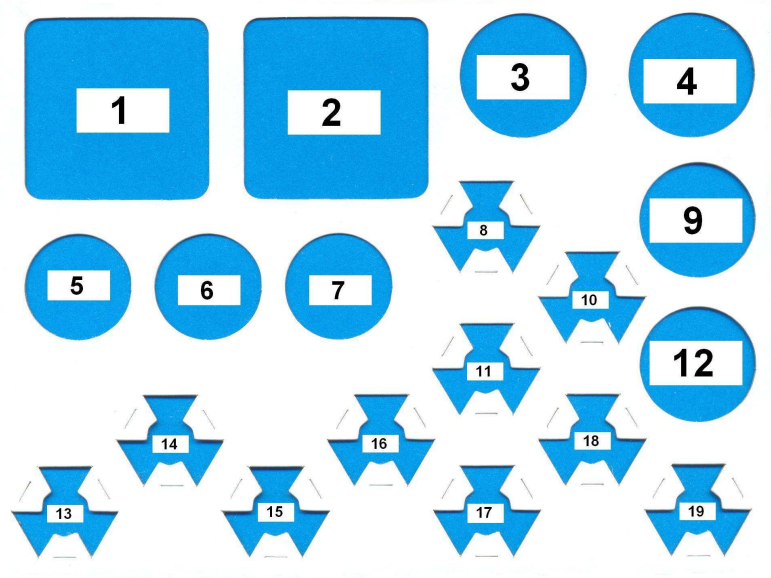
1. Prepare samples according to Chapter 8 (Sample treatment) and prepare reagents according to Chapter 9 (Preparation of reagents).
   Microtiter plate is ready-to-use, do not wash.
2. Pipette 100 µl of zero standard in duplicate (wells A1, A2).
   Pipette 50 µl of zero standard in duplicate (wells B1, B2).
   Pipette 50 µl of each of the standard dilutions in duplicate (wells C1,2 to H1,2 i.e. 20, 10, 2, 1, 0.5 and 0.25 ng Streptomycin/ml).
   Pipette 50 µl of each sample solution in duplicate into the remaining wells of the microtiter plate.
3. Add 25 µl of conjugate (Streptomycin-HRP) to all wells, except wells A1 and A2.
4. Add 25 µl of antibody solution to all wells, except wells A1 and A2.
5. Seal the microtiter plate and shake the plate for a few seconds.
6. Incubate for 1 hour in the dark in a refrigerator (2ºC to 8ºC).
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 min. in a dark place at room temperature (20ºC to 25ºC).
10. Add 100 µl of stop solution to each well.
11. Read the absorbance values immediately at 450 nm.

5. KIT CONTENTS

Manual

1 sealed microtiter plate (12 strips, 8 wells each), coated with antibodies to rabbit 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** (lyophilised, blue cap)
6. **Antibody** (lyophilised, yellow cap)
7. not in use
8. **Standard solution 100 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) **2 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, 2000xg)
- Vortex
- Automated microplate washer or 8 channel micropipette 100-300 µl
- Magnetic stirrer
- Microtiter plate shaker
- Siliconised glass test tubes or plastic tubes
- Micropipettes 20-200 µl, 100-1000 µl
- Multipipette with 2.5 ml combitips
- Aluminum foil or parafilm

9. PREPARATION OF REAGENTS

The reagents included in the test-kit are sufficient to carry out at least 96 analyses (including standard analyses). Each standard and sample is analysed in duplicate.

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 100 ng/ml standard solution.

Before starting the test, the reagents should be brought up to ambient temperature.

Microtiter plate
Return unused strips into zip lock 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 100 ng/ml
To prepare standards in the appropriate matrix or to prepare spikes use the standard solution containing 100 ng Streptomycin per ml. Dilute the standard solution in the appropriate matrix to make a dilution range of 20, 10, 2, 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 (Streptomycin-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 store at –20 ºC.

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 store at –20ºC.

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 x in SDB*.
- E.g. pipette 1 ml of urine sample into a clean siliconised glass tube or plastic vial.
- Add 9 ml of 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).

7. SAFETY PRECAUTIONS

- Good laboratory practise should be employed when using this kit. Safety clothing should be worn and contact with skin and/or mucous membranes avoided.

- Do not eat, drink, smoke, store or prepare foods, or apply cosmetics within the designated work area. Do not pipette by mouth.

- The stop solution contains 0.5 M sulphuric acid (H_2SO_4). Do not allow the reagent to get into contact with skin and/or eyes.

- TMB is toxic by inhalation, in contact with skin and if swallowed; observe care when handling the substrate.

- Do not use components past their 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 surface of the wells, prevent damage and dirt.

- All components should be completely dissolved before use. Take special attention to the substrate, which tends to crystallize at temperatures below +4°C.

- Optimal results will be obtained by strict adherence to this protocol. Careful pipetting and washing throughout this procedure is necessary to maintain good precision and accuracy.

- In case of contact of any reagent with eyes or skin, rinse immediately with plenty of water.

8. SAMPLE TREATMENT

8.1 **Milk samples**
- Defat milk by centrifugation for 10 min. 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 min.
- Centrifuge 15 min., 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.

* 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 **Honey and Royal Jelly samples**

8.5.1. Honey diluted
- Weigh 1 g of homogenized honey in a plastic tube
- Add 4 ml of SDB* and mix well using a vortex
- When no clear solution is obtained filtrate the solution through a 0.8 µm filter
- Pipette 1 ml of the mixture into a clean tube and add 4 ml of SDB*
- Mix well using a vortex
- Use 50 µl of the dilution in the ELISA.

The dilution procedure is not applicable for all honey samples nor Royal Jelly. The composition of the honey sample may give background in the ELISA. For these samples we advise the SPE procedure.

A homogenous sample has to be obtained from a representative part of the honey and Royal Jelly.

8.5.2. SPE procedure
- Weigh 1 g of homogenized honey or Royal Jelly into a plastic tube
- Add 9 ml of distilled water, vortex
- Mix head over head, 30 minutes (Rotor)

Activate the cartridge: (Varian MP1-15 mg no. A5321120)
- Add 1 ml Methanol 100%
- Add 1 ml Distilled water

Note: It is important that the cartridge is not allowed to dry completely during activation and prior to sample addition! If the cartridge has become dry, repeat the activation procedure.

Filtrate the royal jelly mixture through an 0,8 filter

Carefully transfer 2 ml diluted honey or 2 ml royal jelly filtrate onto the activated cartridge (flow 1 ml/min.)

Washing procedure
- Wash the cartridge with 2 ml Methanol 100%
- Let the cartridge dry for two minutes

Elution of the Streptomycin
- Pipette 2 ml SDB* onto the cartridge
- Collect eluent
- Let the cartridge dry for two minutes
- Diluted the eluate 1:1 with SDB*
- Pipette 50 µl into the wells of the ELISA 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).