MULTI-SULFONAMIDES ELISA

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A competitive enzyme immunoassay for
screening and quantitative analysis of a
broad range of sulfonamides
in various matrices

EUROPROXIMA MULTI-SULFONAMIDES ELISA

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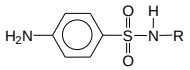
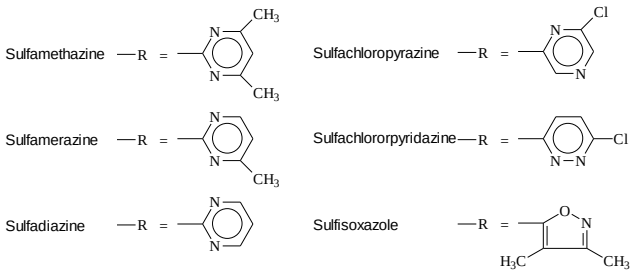
BRIEF INFORMATION

The multi-sulfonamides ELISA is a competitive enzyme immunoassay for the screening and quantitative analyses in various matrices on the presence of a broad range of sulfonamides. The ELISA kit contains a 96-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 sulphonamides from different matrices are included in the kit manual.

1. INTRODUCTION



General structure of sulfonamides



Sulfonamides are a group of synthetic drugs of which the molecules share the p-aminobenzenesulfonamide moiety. They act as competitive antagonists of p-amino-benzoic acid, an essential precursor for a lot of bacteria and protozoon of the essential folic acid. Sulfonamides are antibiotics that are widely used in veterinary and human medicine against bacteria and protozoon (coccidian. Sulfonamides which are retained in food can result in allergic or toxic reactions in for these compounds sensitive consumers. Also, there are general concerns that the widespread use of antibiotics may contribute to antibiotic resistance in pathogenic organisms. Normally, tissue residues in animals are controlled by withdrawing the drug from feed before slaughter. The concentrations of the sulfonamides are then presumed to deplete to less than the maximum residue level (MRL). However, due to contaminated food or failure to observe the withdrawal period, a number of animals are reaching slaughterhouses with a substantially excessive amount of drugs still present in their tissues.

The multi-sulfonamide ELISA can be used to detect residues in tissue, milk, honey, eggs, urine and shrimps. The following sulfonamides can be detected far below the MRL of 100 µg/kg stated by the EC: sulfamethazine, sulfadiazine, sulfamerazine, sulfisoxazole, sulfachloropyridazine and sulfachloropyrazine.

2. PRINCIPLE OF THE MULTI-SULFONAMIDES ELISA

The kit is based on a microtiter plate (12 strips, 8 wells each), precoated with rabbit antibodies to mouse IgG. Specific antibodies (monoclonal mouse anti-Sulfonamide), a horseradish peroxidase labelled sulfonamide (enzyme conjugate) as well as Sulfamethazine 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 antibodies and at the same time free sulfonamides (in the standard solution or in the sample) and enzyme conjugate compete for the specific antibody binding sites (competitive enzyme immunoassay).
After an incubation time of one hour, the non-bound (enzyme labelled) reagents are removed in a washing step. The amount of bound enzyme conjugate is visualised by the addition of a substrate/chromogen solution (tetramethylbenzidine, TMB). Bound enzyme 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 photo metrically at 450 nm and is inversely proportional to the sulfonamides concentration in the sample.

3. SPECIFICITY AND SENSITIVITY

The multi-sulfonamide ELISA utilises antibodies raised in mouse against a protein conjugated sulfonamide. The reactivity pattern of the monoclonal antibody with the tested sulfonamides is:
Cross-reactivity:

Sulfamethazine	100%	Sulfadoxine	<1%
Sulfamerazine	108%	Sulfaguanidine	<1%
Sulfachloropyrazine	97%	Sulfamethoxazole	<1%
Sulfisoxazole	99%	Sulfamethoxydiazine	<1%
Sulfadiazine	68%	Sulfapyridine	<1%
Sulfachloropyridazine	64%	Sulfanilamide	<1%
Sulfathiazole	7%	Sulfacetamide	<1%
Sulfamethizole	5.3%	Sulfaquinoxaline	<1%
Sulfamethoxypyridazine	1.7%	Sulfadimethoxine	<1%
Sulfatroxazole	<1%	N_4-acetyl-sulfadiazine	35%

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) is determined under optimal conditions. Cut-off criteria need critical consideration.

LOD (ppb)						
Procedure	Tissue 8.1	Milk 8.3	Egg 8.1	Honey 8.4	Urine 8.2	Shrimps 8.5
Sulfamethazine	4	< 2.5	3	2	5	0.13

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 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:

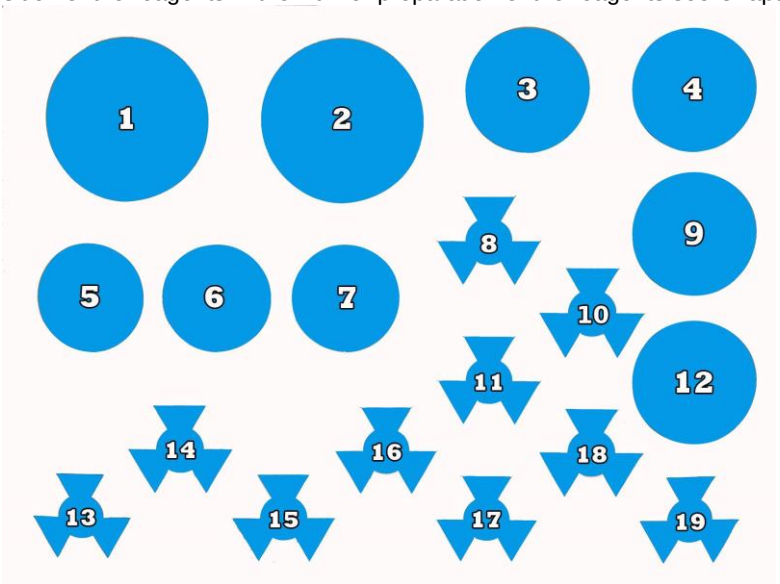
- A blue colouring of the chromogen solution before transferring it into the wells.
- A weak or absent colour reaction of the maximum binding (zero standard) (E450nm < 0.8).

5. KIT CONTENTS

Manual

One sealed microtiter plate (12 strips, 8 wells each), coated with antibodies directed against mouse-IgG. 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** (lyophilised, blue cap)
6. **Antibody** (lyophilised, yellow cap)
7. not in use
8. **Standard solution 100 ng/ml** (1ml, Ready-to-use)
9. not in use
10. not in use
11. not in use
12. not in use
13. **Zero standard solution 1** (2ml, Ready-to-use)
14. **Standard solution 2** (1ml, Ready-to-use) **0.125 ng/ml**
15. **Standard solution 3** (1ml, Ready-to-use) **0.25 ng/ml**
16. **Standard solution 4** (1ml, Ready-to-use) **0.5 ng/ml**
17. **Standard solution 5** (1ml, Ready-to-use) **0.8 ng/ml**
18. **Standard solution 6** (1ml, Ready-to-use) **2.5 ng/ml**
19. **Standard solution 7** (1ml, Ready-to-use) **5.0 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, 3000 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
- Glass test tubes (10 – 15 ml)
- Micropipettes 20 – 200 µl, 100 – 1000 µl
- Multipipette with 2.5 ml combitips
- Aluminium foil or parafilm
- Ethyl acetate
- Iso-octane
- Trichloromethane
- Na_2HPO_4
- KH_2PO_4
- NaCl
- Methanol

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 Tissue and egg samples

- Cut a representative amount of tissue (meat, liver, kidney or egg) sample in fine parts
- Homogenize this sample using a homogenizer, e.g. by use of an Ultra Turrax or a stomacher
- Weigh 1 g finely cut and subsequently homogenized sample into a tube
- Add 5 ml of ethyl acetate
- Mix well with a vortex followed by mixing head over head for at least half an hour
- Centrifuge (5 minutes, 2000 x g)
- Pipette an amount of 1 ml of supernatant (ethyl acetate) into a tube
- Evaporate 1 ml to dryness under a mild stream of nitrogen at 50°C
- Dissolve the residue in 1 ml of phosphate buffered saline (PBS, see chapter 9)
- Defat by adding 1 ml of iso-octane/trichloromethane (2:3; v/v)
- Mix using a vortex and centrifuge (5 minutes, 2000 x g)
- Pipette 100 µl of supernatant into a tube and add 300 µl of PBS
- Use 50 µl portions in the ELISA.

N.B. Instead of iso-octane/trichloromethane, n-hexane can be used. When iso-octane/trichloromethane is used, pipette 100 µl of the upperlayer, when n-hexane is used, take 100 µl of the layer underneath

8.2 Urine samples

- Homogenize the urine samples
- Pipette 1 ml of the homogenized sample into a tube
- Add 5 ml of ethyl acetate
- Mix well with a vortex followed by mixing head over head for at least 15 minutes
- Centrifuge (5 minutes, 2000 x g)
- Pipette an amount of 1 ml of supernatant (ethyl acetate) into a tube
- Evaporate 1 ml to dryness under a mild stream of nitrogen at 50°C
- Dissolve the residue in 1 ml of phosphate buffered saline (PBS, see chapter 9)
- Pipette 100 µl of the dissolved residue into a tube and add 300 µl of PBS
- Use 50 µl portions in the ELISA.

8.3 Milk samples
- Homogenize the milk samples
- Pipette 1 ml of the homogenized sample into a tube
- Add 5 ml of ethyl acetate mix carefully head over head 2 minutes (when shaking is to rigorous, the extract becomes jelly)
- Centrifuge (5 minutes, 2000 x g)
- Evaporate 1 ml to dryness under a mild stream of nitrogen at 50°C
- Dissolve the residue in 1 ml of phosphate buffered saline (PBS, see chapter 9)
- Defat by adding 1 ml of iso-octane/trichloromethane (2:3 v/v)
- Mix using a vortex and centrifuge (5 minutes, 2000 x g)
- Pipette 100 µl of the supernatant into a tube and add 300 µl of PBS
- Use 50 µl portions in the ELISA.

8.4 Honey samples
- Weigh 1 g of the homogenised sample into a tube
- Add 3 ml of 0.1 M HCL and mix using a vortex
- Incubate during one hour at 20°C to 25°C
- Add 3 ml of Sample Equilibration Buffer (5000 SEB)*
- Mix using a vortex
- Add 5 ml of ethyl acetate.
- Mix well with a vortex followed by mixing head over head for at least 15 minutes.
- Centrifuge (5 minutes, 2000 x g).
- Pipette 3 ml of supernatant (ethyl acetate) into a tube
- Evaporate to dryness under a mild stream of nitrogen at a temperature of maximally 50°C.
- Dissolve the residue in 1.2 ml of phosphate buffered saline (PBS, see chapter 9)
- Defat by the addition of 1 ml of iso-octane / trichloromethane (2:3 / V:V)
- Mix using a vortex
- Centrifuge (5 minutes, 2000 x g).
- Pipette 100 µl from the upper layer and add 300 µl of PBS.
- Use 50 µl portions in the ELISA.

N.B. Instead of iso-octane/trichloromethane, n-hexane can be used. When iso-octane/trichloromethane is used, pipette 100 µl of the upperlayer, when n-hexane is used, take 100 µl of the layer underneath

8.5 Shrimp samples
- Weigh 2 g finely cut and subsequently homogenised sample into a polypropylene tube.
- Vortex
- Add 5 ml of ethyl acetate
- Mix well on a vortex followed by mixing head over head for at least 15 minutes
- Centrifuge (5 minutes, 2000 x g)
- Pipette an amount of 2 ml of supernatant (ethyl acetate) into a glass tube
- Evaporate to dryness under a mild stream of nitrogen at 50ºC
- Dissolve the residue in 0.8 ml of phosphate buffered saline (PBS, see chapter 9)
- Defat by adding 1 ml of n-hexane
- Mix using a vortex and centrifuge (5 minutes, 2000 x g)
- Remove the upper layer (n-hexane)
- Use 50 µl portions in the ELISA.

* Sample Equilibration Buffer can be ordered at R-Biopharm Nederland B.V.(see chapter 13).

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 required (1 ml concentrated rinsing buffer + 19 ml distilled water).

Conjugate solution
Reconstitute the vial of lyophilised conjugate with 4 ml of dilution buffer, mix thoroughly and keep in the dark until use. The reconstituted compound can be stored in a refrigerator (2°C to 8°C) for maximally one week. For longer storage make aliquots and freeze at – 20°C.

Antibody solution
Reconstitute the vial of lyophilised antibodies with 4 ml of dilution buffer, mix thoroughly and keep in the dark until use. The reconstituted compound can be stored in a refrigerator (2°C to 8°C) for maximally one week. For longer storage make aliquots and freeze at – 20°C.

Standard solution 100 ng/ml
This standard solution is for spiking and is ready to use.

Substrate/chromogen solution
The substrate/chromogen solution (ready-to-use) tends to precipitate at 4°C. Take care that this vial is at room temperature and mix the content well before pipetting into the wells. Avoid direct (sun) light.

Phosphate Buffered Saline (PBS)
PBS is not provided in the kit. Prepare this buffer as follows:
[Quantities indicated are for 1 liter buffer.]

Na_2HPO_4	0.77 g
KH_2PO_4	0.18 g
NaCl	8.94 g
Distilled water add	1000 ml

pH 7.4 ± 0.2

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 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
   Do not wash the ready-to-use microtiter plate.

2. Pipette 100 µl of the zero standard in duplicate (wells H1, H2, blank).
   Pipette 50 µl of the zero standard in duplicate (wells A1, A2, maximal signal).
   Pipette 50 µl of each of the Sulfamethazine standard solutions in duplicate (wells B1,2 to G1,2 i.e. 0.125, 0.25, 0.5, 0.8, 2.5 and 5 ng/ml).

3. Pipette 50 µl of each prepared sample solution in duplicate into the remaining wells of the microtiter plate

4. Add 25 µl conjugate (sulfonamide-HRP) to all wells, except wells H1 and H2.

5. Add 25 µl antibody solution to 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 the plate for 1 hour in the dark at 4°C (2°C to 8°C).

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

9. Add 100 µl substrate/chromogen solution to all wells.

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

11. Add 100 µl stop solution to each well.

12. Immediately after adding the stop solution, read the absorbance values at 450 nm.

11. INTERPRETATION OF RESULTS

In general, the O.D. values of the samples, that are measured in this ELISA test, are converted into corresponding concentrations in the following way:

The mean optical density (O.D.) value of the blank wells is subtracted from the individual O.D. of the wells containing the standards and the samples. Then these O.D. values of the standards and the samples (mean values of the duplicates) are divided by the mean O.D. value of the zero standard 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.
The values calculated for the standards are entered in a semi-logarithmic plot against the concentration of the analyte.

Alternative for calibration curve:
The values of absorption (logit) calculation of the standards are plotted on Y-axis versus the analyte equivalent concentration on a logarithmic X-axis

As customer-friendly options R-Biopharm offers 2 software programs that can be used for the interpretation of the ELISA test:

- RIDASOFT®Win.NET Food & Feed (art. nr. Z9996FF). In this software program all R-Biopharm ELISA tests (including EuroProxima) are preprogrammed in the database. A list of compatible readers is available on request.
- Simplefit. This Excel based program is developed to calculate the results of all EuroProxima tests.

8.1 Tissue and egg samples
The Sulfonamide equivalents, as read from the standard curve, have to be multiplied by a factor 20 (0.05 g sample/ml buffer) to obtain the sulphonamides content in tissue and egg samples.

8.2 Urine samples
The Sulfonamide equivalents, as read from the standard curve, have to be multiplied by a factor 20 (0.05 g sample/ml buffer) to obtain the sulphonamides content in urine samples.

8.3 Milk samples
The Sulfonamide equivalents, as read from the standard curve, have to be multiplied by a factor 20 (0.05 g sample/ml buffer) to obtain the sulphonamides content in milk samples.

8.4 Honey samples
The Sulfonamide equivalents, as read from the standard curve, have to be multiplied by a factor 8 (0.6 g sample/1.2 ml buffer followed by a 1:4 dilution) to obtain the sulphonamides content in honey samples.

8.5 Shrimp samples
The sulfonamide equivalents can be directly read from the standard curve (dilution factor is 1) to obtain the sulfonamides content in shrimp samples.

12. LITERATURE

W. Haasnoot, F. Cohen, J. du Pré, G. Cazemier, A. Kemmers-Voncken, M. Bienenmann-Ploum and R. Verheijen. Application of generic monoclonal antibodies against sulfonamides in optical biosensors. In: Proceedings of the Euroresidue IV Conference, 8-10 May 2000, Veldhoven, the Netherlands, pp 501-505.

EMEA; The European Agency for the Evaluation of Medicinal Products. EMEA/MRL/026/95. Summary report sulfonamides (2).

13. ORDERING INFORMATION

For ordering the multi-sulfonamide ELISA kit, please use cat. code 5101SULM.
For ordering the Sample Equilibration Buffer, please use cat. code 5000SEB

14. REVISION HISTORY

A new sample preparation for shrimps has been introduced (chapter 8) with the associated new data for LOD (chapter 3) and calculation factor (chapter 11).

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