SEM ELISA

5091SEM[7]09.23

A competitive enzyme immunoassay for screening and quantitative analysis of SEM

EUROPROXIMA SEM ELISA

A competitive enzyme immunoassay for screening and quantitative analysis of SEM

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BRIEF INFORMATION

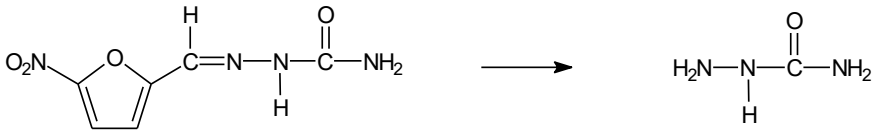
The SEM ELISA is a competitive enzyme immunoassay for the measurement of SEM in various matrices. With this ELISA-kit 96 analyses can be performed. Samples and standards are measured in duplicate which means that a total of 41 samples can be analyzed.
The SEM ELISA contains all reagents to perform the test. Reagents for sample preparation are not included.

1. INTRODUCTION

The nitrofurans are a group of synthetic broad-spectrum antibiotics, which have been widely and effectively used for the prevention and treatment of gastrointestinal infections caused by *Escherichia coli*, *Salmonella spp.*, *Mycoplasma spp.*, *Coccidia spp.*, coliforms and other protozoa in animal production and aquaculture. Moreover, nitrofurans have been employed as growth promoters in livestock.
The four major nitrofurans are furazolidone, furaltadone, nitrofurantoin and nitrofurazone. They have been banned in the EU for use as veterinary drugs, due to their toxic an suspected carcinogenic and mutagenic properties (Commission Regulation 1442/95). In 2003 a definitive MRPL (Minimum Required Performance Limit) was set at 1 ng/g (ppb) in the EU for all four of the above mentioned nitrofurans in poultry and aquaculture products (Commission Decision 2003/181/EC).
Various studies have demonstrated that the nitrofuran parent molecules are rapidly metabolised by animals and that their *in vivo* stability is not longer than a few hours. As a result, persistent protein-bound residues are formed. Unlike the parent molecules, these protein-bound metabolites are stable and persistent in the body. It is possible to free these residues from proteins by acid hydrolysis. Testing for the presence of nitrofurans is thus equivalent to testing for the presence of a part of the parent molecule, i.e. the free residue. Nitrofuran residues are found after administration of furaltadone (3-amino-5-morpholinomethyl-2-oxazolidinone = AMOZ), furazolidone (3-amino-2-oxazolidinone = AOZ), nitrofurantoin (1-aminohydantoin = AHD) and nitrofurazone (semicarbazide = SEM).

Note: For the determination of AMOZ, AOZ and AHD we refer to the EuroProxima ELISAs 5091AMOZ, 5091AOZ and 5091AHD, respectively.

PARENT DRUG STABLE METABOLITE



Nitrofurazone Semicarbazide (SEM)

2. PRINCIPLE OF THE SEM ELISA

The microtiter plate based SEM ELISA consists of one precoated plate (12 strips, 8 wells each). Horseradish peroxidase (-HRP) labeled SEM and standard solution or sample are added to the wells. Free SEM from the samples or standards and SEM-HRP conjugate compete for the specific antibody binding sites (competitive enzyme immunoassay).
After an incubation step of 30 minutes, the non-bound reagents are removed in a washing step. The amount of bound SEM-HRP conjugate is visualized by the addition of a substrate/chromogen solution (tetramethylbenzidine, TMB). Bound SEM-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 SEM concentration in the sample.

3. SPECIFICITY AND SENSITIVITY

The SEM ELISA utilizes antibodies raised in mouse against protein conjugated SEM. The reactivity pattern of the antibody is:

Cross- reactions:	SEM	100%
	AMOZ	< 0.01%
	AHD	< 0.01%
	AOZ	< 0.01%

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.

Matrix	Procedure	LOD ppb
Milk	8.1	0.1
Honey	8.2	0.2
Egg	8.3	0.1
Egg (powder)	8.4	0.1
Shrimps	8.5	0.1
Tissue	8.6	0.2
Fish	8.7	0.2
Liver	8.8	0.2
Urine	8.9	0.3

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:

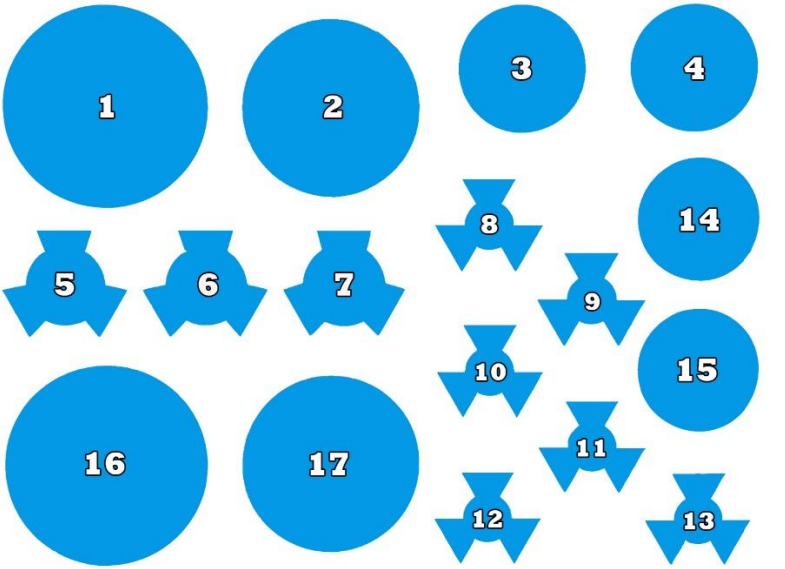
- A blue colouring of the chromogen solution before transferring it into the wells.
- A weak or no colour reaction in the zero standard wells (E450nm < 0.8).

5. KIT CONTENTS

Manual

One sealed (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. **Sample dilution buffer** (40 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 solution** (100 µl, 100 times concentrated)
6. **SEM-stock solution** (1 ml 100 ng/ml for spiking samples)
7. **Zero Standard** (2 ml, ready-to-use)
8. **Standard solution 1** (1 ml, ready-to-use) **0.056 ng/ml SEM-NP**
9. **Standard solution 2** (1 ml, ready-to-use) **0.167 ng/ml SEM-NP**
10. **Standard solution 3** (1 ml, ready-to-use) **0.5 ng/ml SEM-NP**
11. **Standard solution 4** (1 ml, ready-to-use) **1.5 ng/ml SEM-NP**
12. **Standard solution 5** (1 ml, ready-to-use) **4.5 ng/ml SEM-NP**
13. Not in use
14. **Dilution buffer** (15 ml ready-to-use)
15. Not in use
16 Not in use
17. Not in use

6. EQUIPMENT AND MATERIALS REQUIRED BUT NOT PROVIDED

- 4 ml glass tubes
- 15 ml tubes with screw cap
- Scales and weighing vessels
- Gloves
- Fume hood
- Homogeniser (vortex, mixer)
- Centrifuge (2000 x g)
- Automated microtiter plate washer or 8-channel micropipette 100 – 300 µl
- Microtiter plate shaker
- Microtiter plate reader with 450 nm filter
- Glass test tubes (10 – 15 ml)
- Micropipettes, 100 – 1000 µl
- Multipipette with 2.5 ml combitips
- Aluminium foil or parafilm
- Distilled water (bidest)
- Ethyl acetate
- Dimethyl sulfoxide (DMSO)
- n-Hexane
- 1 M HCl
- 2-nitrobenzaldehyde
- 0.25 M K_2HPO_4 (43.5 gram/L distilled water)
- 1 M NaOH (40 gram/l distilled water)

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

Each matrix requires its own specific derivatisation step followed by a general extraction procedure.

For optimal recovery of the derivatisation procedure, each laboratory has to run its own in-house validation. For individual nitrofurans and matrices we propose a concentration of the derivative reagent as well as a derivatisation time. However, these proposals are guidelines and have to be tested for optimal results in each individual lab.

Derivatisation

8.1 Milk

Centrifuge cold milk samples for 10 minutes at 2000 x g at 4°C.
Remove the upper fat layer using a spatula. Use 1 ml defat sample for derivatisation.

Derivation

- Mix gently 1 ml of the homogenized defatted milk sample with 4 ml double distilled water, 0.5 ml of 1 M HCL and 250 µl derivative reagent* in a 15 ml centrifuge tube.
- Mix carefully head over head for 1 minute (when shaking is too rigorous, the extract becomes jelly).
- Incubate at 37ºC for 1 hour.

8.2 Honey

- Mix 1 gram of the honey sample with 4 ml double distilled water, 0.5 ml of 1 M HCL and 250 µl derivative reagent* in a 15 ml centrifuge tube.
- Mix head over head until the honey is completely dissolved approximately 5 minutes.
- Incubate at 37ºC 1 hour.

8.3 Egg

- Mix gently 1 gram of the homogenized whole egg sample with 4 ml double distilled water, 0.5 ml of 1 M HCL and 150 µl derivative reagent* in a 15 ml centrifuge tube.
- Mix carefully head over head for 1 minute (when shaking is too rigorous, the extract becomes jelly).
- Incubate at 37ºC 1 hour

8.4 Egg powder

- Weigh 1 gram of egg powder in a tube, add 5 ml of water, mix until a homogeneous solution.
- Mix 1 ml of the homogenized sample with 4 ml double distilled water, 0.5 ml of 1 M HCl, and 150 µl derivative reagent*.
- Mix carefully head over head for 1 minute (when shaking is too rigorous, the extract becomes jelly).
- Incubate at 37ºC 1 hour

8.5 Shrimps
- Mix 1 gram of the homogenized shrimps sample with 4 ml double distilled water, 0.5 ml of 1 M HCL and 100 µl derivative reagent* in a 15 ml centrifuge tube.
- Mix head over head for 5 minutes.
- Incubate at 37ºC for 1 hour, or 37°C for 3 hours, or 37°C overnight.

8.6 Tissue
- Mix 1 gram of the homogenized sample with 4 ml double distilled water, 0.5 ml of 1 M HCL and 200 µl derivative reagent* in a 15 ml centrifuge tube.
- Mix head over head for 5 minutes.
- Incubate at 37ºC for 1 hour, or 37°C for 3 hours, or 37°C overnight.

8.7 Fish
- Mix 1 gram of the homogenized sample with 4 ml double distilled water, 0.5 ml of 1 M HCL and 250 µl derivative reagent* in a 15 ml centrifuge tube.
- Mix head over head for 5 minutes.
- Incubate at 37ºC 1 hour

8.8 Liver
- Mix 1 gram of the homogenized sample with 4 ml double distilled water, 0.5 ml of 1 M HCL and 250 µl derivative reagent* in a 15 ml centrifuge tube.
- Mix head over head for 5 minutes.
- Incubate at 37ºC 1 hour

8.9 Urine
- Centrifuge the urine samples 5 minutes at 2000xg.
- Mix 1 ml of the clear urine sample with 4 ml double distilled water, 0.5 ml of 1 M HCL and 200 µl derivative reagent* in a 15 ml centrifuge tube.
- Mix head over head for 5 minutes.
- Incubate at 37ºC 1 hour

* Derivative reagent
  10 mM 2-nitrobenzaldehyde in Dimethylsolfoxid (DMSO)
  This solution has to be prepared directly before use.
  i.e. dissolve 15.2 mg 2-nitrobenzaldehyde in 10 ml DMSO.

Note: 2-nitrobenzaldehyde is sensitive to oxidizing agents. Therefore, keep away from open air or other incompatible substances. Keep container tightly closed when not in use. Do not use material that has been expired.

Extraction procedure
- Add to the derivatized sample 2.5 ml 0.25 M K_2HPO_4 , 0.4 ml of 1 M NaOH and 5 ml ethyl acetate.
- Mix carefully head over head (when shaking too rigorous, the extract becomes jelly), for 1 minute.
- Centrifuge for 10 minutes at 2000 x g.
- Transfer 2.5 ml of the ethyl acetate layer (upper layer) into a 4 ml glass tube.
- Evaporate to dryness at 50ºC under a mild stream of nitrogen.
- Dissolve the residue in 1 ml n-hexane. Add 1 ml sample dilution buffer.
- Vortex for 1 minute.
- Centrifuge for 10 minutes at 2000 x g.
- Remove the upper, Hexane layer.
- If not separate to two phases, heat the above sample for about 3 minutes (80ºC~100ºC)
- Centrifuge for 10 minutes at 2000 x g.
- Use 50 µl of the aqueous phase in the ELISA.

9. PREPARATION OF REAGENTS

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 +8ºC. Prepare reagents freshly before use.

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. For each strip 20 ml of diluted rinsing buffer is used (1 ml concentrated rinsing buffer + 19 ml distilled water).

Dilution buffers
This ELISA contains two dilution buffers, both ready to use provided. The dilution buffer for the conjugate (chapter 5 no. 9) and sample dilution buffer (chapter 5 no. 1) for dissolving of the samples.

Conjugate solution (100 µl)
The conjugate (SEM-HRP) is delivered 100x concentrated. Spin down the conjugate in the vial by a short centrifugation step (1 minute, 1000 x g). Add 10 µl of the concentrated conjugate solution to 1 ml of dilution buffer. Per 2 x 8 wells 800 µl of diluted conjugate is required. Store unused concentrated conjugate at +2ºC to +8ºC.

Substrate/chromogen solution
The substrate/chromogen solution (ready-to-use) tends to precipitate at +4ºC. Take care that this vial is at room temperature when used (keep in the dark) and mix the content before pipetting into the wells.

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 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 and prepare reagents according to Chapter 9.

2. Pipette 100 µl of the zero standard in duplicate (wells G1, G2, blank).
   Pipette 50 µl of the zero standard in duplicate (wells A1, A2, maximal signal).
   Pipette 50 µl of each of the standard solutions in duplicate (wells B1,2 to F1,2 i.e. 0.056, 0.167, 0.5, 1.5 and 4.5 ng/ml).

3. Pipette 50 µl of each sample solution in duplicate into the remaining wells of the microtiter plate (41 samples; 82 wells).

4. Pipette 50 µl of conjugate into all wells except the blank G1 and G2.

5. Seal the microtiter plate and shake the plate for a few seconds on a microtiter plate shaker.

6. Incubate for 30 minutes in the dark at room temperature (20ºC to 25ºC)

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 15 minutes 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.

11.INTERPRETATION OF RESULTS

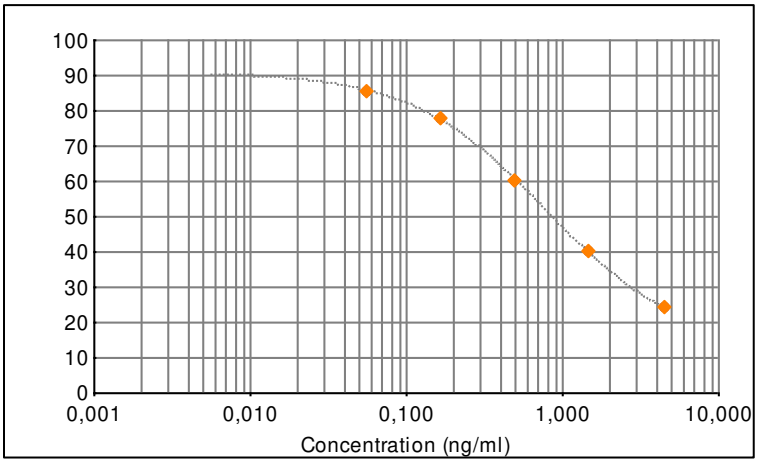
Subtract the mean optical density (O.D.) of the wells G1 and G2 (Blank) from the individual O.D. of the wells containing the standards and the samples.

The O.D. values of the five 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 value of absorption (logit) calculation of the standards are plotted on Y-axis versus the analyte equivalent concentration on a logarithmic X-axis



Example of a calibration curve

The amount of SEM in the samples is expressed as SEM equivalents. The SEM equivalents in the samples (ng/ml) corresponding to the percentage maximal absorbance of each extract can be read from the calibration curve.

All matrices have the same calculation factor.
The SEM equivalents read from the calibration curve have to be multiplied by 2.

12. LITERATURE

Joint Expert Committee on Food Additives (JECFA), Monographs and Evaluations Web site, http://www.inchem.org/documents/jecfa/jecmono/v31je07.htm, 31 October 2006

Van Koten-Vermeulen, J.E.M., Wouters, M.F.A., Van Leeuwen, F.X.R. Report of the 40th Meeting of the Joint FAO/WHO Expert Committee on Food Additives, World Health Organisation, Geneva 1993, 85-123.

European Union Commission Regulation 1442/95, *Off. J. Eur. Commun.* 1995, **L143**, 26-30

European Union Commission Regulation 2901/93, *Off. J. Eur. Commun.* 1993, **L264**, 1-4

Nouws, J.F.M. , Laurensen, J. *Vet. Q.* 1990, **12**, 56-59

McCracken, R.J., Blanchflower, W.J., Rowan, C., McCoy, M.A., Kennedy, D.G. *Analyst* 1995, **120**, 2347-2351

Hoogenboom, L.A.P., Tomassini, O., Oorsprong, M.B.M., Kuiper, H.A. *Chem. Toxicol.* 1991, **29**, 185-191

McCracken, R.J., Kennedy, D.G. *Chromatogr. A* 1997, **771**, 349-354

Gottschall, D.W., Wang, R. J. *Agric. Food Chem.* 1995, **43**, 2520-2525

13. ORDERING INFORMATION

For ordering the SEM ELISA kit, please use cat. code 5091SEM.

14. REVISION HISTORY

The manual is adapted to the use of five standards instead of six. See chapter 5 and further.

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