AgraQuant® Nitrofuran (SEM) Assay

Order No.: COKDA1400
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Nitrofuran (SEM)
Nitrofurans are synthetic broad-spectrum antibiotics used for various bacterial and protozoan infections in fish, shrimp, bees, swine, cattle and poultry. It is also used as a growth promoter. Nitrofurazone is metabolised rapidly \textit{in vivo}, forming SEM tissue bound residues in livestock.

Short Instructions:

1. Pipette 50 µL each of the serial calibrator into wells.
2. Pipette 50 µL samples into the remaining wells.
3. Pipette 50 µL conjugate solution into all wells.
4. Seal plate and shake plate gently to mix well. \textbf{Incubate plate for 30 minutes}.
5. Wash plate 3 times with diluted wash buffer.
6. Tap dry washed wells.
7. Pipette 150 µL substrate solution into wells. Seal plate and incubate plate for 15 minutes in the dark.
8. Add 50 µL stop solution into the wells.
9. Read the strips with ELISA reader \textbf{using 450nm filter and 630nm differential filter}.

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**Performance Characteristics:**

**LOD:**
- Shrimp: 0.22 ppb
- Chicken: 0.21 ppb
- Urine: 0.43 ppb

**LOQ:**
- Shrimp: 0.27 ppb
- Chicken: 0.27 ppb
- Urine: 0.48 ppb

**Quantitation Range:**
- Shrimp: 0.3 – 3 ppb
- Chicken: 0.3 – 3 ppb
- Urine: 0.5 – 3.75 ppb

(For quantitation of samples above the upper limit, samples should be diluted so that the diluted sample results are in the range indicated above).

**Cross-Reactivities to:** Nitrofuran (SEM) (100%)

**No Cross-Reactivities to:** Nitrofuran (AMOZ), Nitrofuran (AOZ), Nitrofuran (AHD) & Nitrofuran (DNSH)
Solution Preparation

Wash Buffer
Dilute wash buffer concentrate 1:20 with distilled water to the required amount before using (e.g. add 20 mL of concentrated wash buffer to 380 mL of distilled water to make 400 ml diluted wash buffer). The diluted wash buffer may be stored in the fridge for up to five weeks.

Sample Preparation / Extraction

Chicken and Shrimp Samples (Dilution Factor: x2)
1. Homogenise at least 10 g of tissue sample.
2. Weigh 1 g of the homogenised sample and add 4 mL of distilled water to the sample, followed by 0.5 mL of 1 M hydrochloric acid (HCl) and 100 μL of 10 mM 2-Nitrobenzoic aldehyde (dissolved in dimethylsulfoxide [DMSO]). Mix well by vortexing for 30 seconds.
3. Incubate at 55 °C for 2 hrs and 15 mins.
4. After that, dispense 5 mL of 0.1 M di-potassium hydrogen phosphate (K2HPO4) and 0.4 mL 1 M sodium hydroxide (NaOH) into each sample. Finally, pipette 5 mL of ethyl acetate into every sample and shake or vortex vigorously for 30 seconds.
5. Centrifuge for 10 mins at 3000 g at room temperature.
6. Transfer 2.5 mL of the upper ethyl acetate layer into a clean glass tube.
7. Dry the solvent under a gentle stream of nitrogen at 50 °C.
8. Dissolve the residue in 1 mL of n-hexane followed by 1 mL of dilution buffer. Mix well by vortexing for 30 seconds.
9. Centrifuge for 10 mins at 3000 g at room temperature.
10. Use 50 μL of the lower aqueous phase for the assay analysis.
Urine Samples (Dilution Factor: x 2.5)

1. Centrifuge urine samples at 3000 g for 10 minutes.
2. Pipette 1.0 mL of the urine sample and add 0.25 mL of 1 M hydrochloric acid (HCl), followed by 5.0 mL of ethyl acetate.
3. Vortex sample vigorously for 1 min.
4. Centrifuge sample 3000 g for 10 mins
5. Remove the upper ethyl acetate layer as much as possible and pipette 0.5 mL of the urine sample (bottom layer) into a new centrifuge tube.
6. Add 2 mL of distilled water to the sample, followed by 0.1 mL of 1 M hydrochloric acid (HCl) and 0.1 mL of 10 mM 2-Nitrobenzoic aldehyde (dissolved in dimethylsulfoxide [DMSO]). Mix well by vortexing for 1 min.
7. Incubate at 55 °C for 2 hours and 15 mins.
8. After that, dispense 2.5 mL of 0.1 M di-potassium hydrogen phosphate (K$_2$HPO$_4$) and 0.2 mL of 1 M sodium hydroxide (NaOH) into each sample. Finally, pipette 2.5 mL of ethyl acetate into every sample and shake or vortex vigorously for 1 min.
9. Centrifuge for 10 minutes at 3000 g at room temperature.
10. Transfer 1.25 mL of the upper ethyl acetate layer into a clean glass tube.
11. Dry under a gentle stream of nitrogen at 50 °C.
12. Dissolve the residue in 0.5 mL of dilution buffer. Mix well by vortexing for 30 seconds.
13. Use 50 μL of the lower aqueous phase for the assay analysis.
Assay Procedure in Detail

Note: All reagents and kit components must be at room temperature 18-30 °C (64-86 °F) before use. It is recommended that an 8-channel pipette be used for pipetting Substrate and Stop Solution in the assay. Use a single channel pipette to pipette all other reagents. Change pipette tips when pipetting different standards and samples.

1. Allow assay reagents and microtitre plate to equilibrate to room temperature prior to use.
2. Place an appropriate number of Antibody Coated Microwells in a microwell strip holder. Return unused microwells to the aluminium zip lock bag with desiccants and store 2-8 °C for future use.
3. Pipette 50 µL of each of the serial calibrator in duplicate to wells A1 and A2 → E1 and E2.
4. Pipette 50 µL of each prepared sample solution in duplicate into the remaining wells of the microtitre plate.
5. Pipette 50 µL of the SEM-HRP conjugate solution into all the wells.
6. Seal plate with sealing tape.
7. Mix the contents thoroughly, by moving the plate for at least 1 minute.
8. Incubate the plate for 30 minutes at room temperature.
9. Once the incubation is completed, discard all the solutions from the microtitre plate wells.
10. Using a pipette, dispense 300 µL of the diluted wash buffer into each well.
11. Move the plate in a circular motion to rinse the wells. Discard the contents after rinsing.
12. Repeat the wash process (steps 10 to 11) twice more for a total of 3 washes.
13. Upon completion of the last wash step, tap the plate dry onto a clean, lint-free towel to remove as much of the remaining liquid in the wells as possible. Wipe the bottom of the wells with a lint-free towel to remove any liquid on the outside of the wells.
14. Add **150 µL of TMB substrate solution** into all wells and seal the plate. Mix the plate thoroughly and incubate for **15 minutes** in the dark at room temperature.

15. Add **50 µL of stop solution** to all wells. Mix gently before reading. Read the optical density values immediately on a microplate reader at **450 nm measuring wavelength** and **630 nm reference wavelength**.

**Additional Notes:** Do not return unused reagents to their original bottles.

**Interpretation of Results**

1. Using either the unmodified OD values or the OD values expressed as a percentage of the OD of the zero (0) standard, construct a dose-response curve using the five standards. Since the amount of Nitrofuran (SEM) in each standard is known, the unknowns can be measured by interpolation from this standard curve.

2. Results can also be easily calculated using the Romer® Log/Logit spreadsheet that is provided (free of charge) upon request. If the Log/Logit regression model is used for results interpretation, the correlation coefficient (R) of the Log and Logit data sets for calibration curve must be between -0.990 to -1.000.

3. The Nitrofuran (SEM) equivalent concentrations can be read from the calibration curve.
   a) For chicken and shrimp samples, the Nitrofuran (SEM) equivalent concentrations have to be multiplied by a factor of 2.
   b) For urine samples, the Nitrofuran (SEM) equivalent concentrations have to be multiplied by a factor of 2.5.

4. An OD value of less than 0.6 absorbance units for the zero calibrator may indicate deterioration of reagents.
Materials supplied

The reagents provided are sufficient for at least 96 analyses (including the calibrators). Each sample and calibrator should be analyzed in duplicates for average optical density.

- 1 microtitre plate (96 wells, 12 breakable strips) sealed in an aluminium zip lock bag.
- 1 piece of sealing tape
- 5 bottles of Nitrofuran (SEM) calibrators (0 ng/mL, 0.05 ng/mL, 0.15 ng/mL, 0.5 ng/mL and 1.5 ng/mL) (1 mL each).
- 2 bottles of SEM-HRP conjugate solution (6 mL total).
- 1 bottle of dilution buffer, pH 7.5 (100 mL).
- 1 bottle of wash buffer (20x concentrate; 20 mL).
- 1 bottle of TMB substrate solution (20 mL).
- 1 bottle of stop solution (10 mL).
- Instruction manual.
- Certificate of Analysis

Materials required but not supplied

- Ethyl acetate
- 2-Nitrobenzoic aldehyde
- 1 M NaOH
- 0.1 M K$_2$HPO$_4$
- Drying system with N$_2$ gas
- Timer
- Sample tube
- Dimethylsulfoxide (DMSO)
- 1 M HCl
- n-hexane or n-heptane
- Centrifuge
- Pipettes
- Microplate reader
- Vortex mixer

Technical and Background Information

Intended Use
The AgraQuant® Nitrofuran (SEM) Assay is a direct competitive enzyme-linked immunosorbent assay (ELISA) that determines a quantitative level of the presence of Nitrofuran (SEM) in food. It
represents a highly sensitive detection system and is designed for the quantification of Nitrofuran (SEM) in meat (muscle).

**Nitrofuran (SEM)**
Nitrofurans are synthetic broad-spectrum antibiotics used both prophylactically and therapeutically for various bacterial and protozoan infections in fish, shrimp, bees, swine, cattle and poultry, such as gastrointestinal enteritis (caused by *Escherichia coli*, and *Salmonella* spp.), fowl cholera and coccidiosis black-heads. It is also use as a growth promoter. Nitrofurazone is metabolised rapidly *in vivo*, forming SEM tissue bound residues in livestock. The use of nitrofuran antibiotics was prohibited in livestock production in the European Union (EU) in 1995 due to concerns about the carcinogenicity of their residues in edible tissues. In March 2003, EU has established a Minimum Required Performance Limits (MPRL) for nitrofuran related residues detected in poultry meat and aquaculture products at 1.0 µg/kg for each metabolite.

**Assay Principles**
The AgraQuant® Nitrofuran (SEM) Assay is a direct competitive enzyme-linked immunosorbent assay (ELISA) which operates on the basis of competition between the drug / metabolite in the sample and the drug-enzyme conjugate for the limited number of specific antibody binding sites. After 30 minutes incubation, the unbound reagents are removed in a washing step. The enzyme conjugate uses horseradish peroxidase (HRP) as a tracer. The amount of SEM-HP bound is measured by the addition of a chromogen substrate, tetramethylbenzidine (TMB). The bound SEM-HP conjugate converts the colourless TMB substrate solution to a blue-coloured product. The TMB reaction is stopped by the addition of the stop solution, which converts the solution to a yellow-coloured product. The colour intensity is measured at a wavelength of 450 nm with a microplate reader using a reference wavelength of 630 nm. The color intensity is
inversely proportional to the nitrofuran (SEM) concentration in the calibrator or sample.

**Precautions**

1. Store reagents at 2-8 °C (35-46 °F) when not in use, and do not use beyond the expiration date.
2. Adhere to incubation times stated in the procedure. Use of incubation times other than those specified may give inaccurate results.
3. Due to high risk of cross contamination all used instruments have to be cleaned thoroughly before sample preparation.
4. The Stop Solution contains acid. Avoid contact with skin or eyes. If exposed, flush with water.
5. Wear protective gloves and safety glasses when using the kit.
6. Dispose of all materials, containers and devices appropriately after use.
7. Each well is ultimately used as an optical cuvette. Therefore, avoid touching the bottom, outside or inside of the wells.
8. Take note that the TMB substrate solution crystallizes at temperatures below +2 °C and is light sensitive.
9. In the case of the TMB substrate solution changing to a blue colour, avoid using the kit reagents.
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