# Nitrofurazone Metabolite (SEM) ELISA Kit

**Catalog No.:** abx364818

**Size:** 96T

**Storage:** Store at 4°C for up to 6 months. For long term storage, the ELISA plate, Standards and antibody can be stored at -20°C.

**Application:** For quantitative detection of SEM in Serum, Tissue, Liver, Honey, Milk, fish, shrimp, birds.

**Sensitivity:** 0.05 ppb (ng/ml)

**Detection Limit:** Tissue/liver – 0.1 ppb, Honey/milk/casing – 0.1 ppb, Milk powder/egg powder/fodder – 0.1 ppb, Fish and shrimp – 0.15 ppb

**Introduction:** Nitrofurazone is an antimicrobial organic compound belonging to the furan class. Nitrofurazone is indicated for topical use in dogs, cats, and horses, for the treatment or prophylactic treatment of superficial bacterial infections, burns, and cutaneous ulcers. Preparations for treating infections, such as fin rot, in ornamental fish are also still commercially available. The use of Nitrofurazone, or related compounds, in animals raised for human consumption has been strictly banned.

### Principle of the Assay

This kit is based on indirect-competitive enzyme-linked immuno-sorbent assay technology. SEM is pre-coated onto a 96-well plate. The standards and samples and an antibody specific to SEM are added to the wells and incubated. After washing away the unbound conjugates, Horseradish Peroxidase is added to each microplate well. After TMB substrate solution is added only wells that contain SEM will produce a blue colour product that changes into yellow after adding the stop solution. The intensity of the yellow colour is inversely proportional to the SEM amount bound on the plate. The O.D. absorbance is measured spectrophotometrically at 450 nm in a microplate reader, and then the concentration of SEM can be calculated.

### Kit components

1. One pre-coated 96 well plate
2. Standard: 1 ml each of:
   0 ppb, 0.05 ppb, 0.15 ppb, 0.45 ppb, 1.35 ppb, 4.05 ppb, 100 ppb
3. Derivatization Reagent: 10ml
4. HRP Conjugate Reagent: 5.5 ml
5. Primary antibody solution: 5.5 ml
6. Substrate reagent A: 6 ml
7. Substrate reagent B: 6 ml
8. Stop solution: 6 ml
9. Wash buffer (20X): 40 ml
10. Re-dissolve buffer (2X): 50 ml

### Materials Required But Not Provided

1. 37°C incubator
2. Microplate reader (450 nm)
3. High-precision pipette and sterile pipette tips
4. Automated plate washer
5. ELISA shaker
6. 1.5 ml EP tubes to prepare samples
7. Distilled water
8. Absorbent filter papers
9. 100 ml and 1 L graduated cylinders
10. Nitrogen evaporator
11. Centrifuge
12. Homogenizer

### Reagents Required But Not Provided

1. Ethyl acetate
2. N-hexane
3. Concentrated HCl
4. Potassium nitroprusside ($K_2Fe(CN)_5(NO)\cdot 2H_2O$)
5. Potassium phosphate dibasic trihydrate (K2HPO4•3H2O)
6. Zinc sulfate ($ZnSO_4\cdot 7H_2O$)
7. Deionized water
8. Sodium Hydroxide

## Protocol

### A. Preparation of sample and reagents

#### 1. Preparation of Sample pretreatment solutions

- **Solution 1 - 0.36 M K2HPO4•3H2O, K2Fe (CN) 5(NO) •2H2O solution (for milk and milk powder sample)**
  Dissolve 11.9g M K2HPO4•3H2O, K2Fe (CN) 5(NO) •2H2O to 100 mL with deionized water.

- **Solution 2 - 1.04 M Zinc Sulfate Solution (*milk, milk powder*)**
  Dissolve 29.8 g of Zinc sulfate to 100ml of deionized water.

- **Solution 3 - 0.1 M Potassium phosphate dibasic trihydrate (K2Fe(CN)5(NO)·3H2O**
  Dissolve 11.4g K2HPO4•3H2O to 500 mL with deionized water.

- **Solution 4 - 1 M HCl solution**
  Dilute 8.6 mL concentrated HCl to 100 mL with deionized water
  .
- **Solution 5 - 1 M NaOH solution**
  Dilute 4g NaOH to 100 mL with deionized water
  .
- **Solution 6- Re-dissolve Solution (1X)** Note: If the sample is aquiform, do not dilute it.
  Dilute the 2× Re-dissolve Buffer with deionized water 2-fold (i.e. dilute 50 ml 2x Re-dissolve buffer in 50 ml Deionized water) to make the 1× Re-dissolve Buffer solution. The 1× solution can be stored at 4°C for up to one month.

- **Solution 7: Wash Buffer**
  Dilute 20×Concentrated Wash Buffer with deionized water. (20×Concentrated Wash Buffer (V): Deionized water (V) = 1:19)

#### 2. Sample pretreatment

Store samples to be assayed within 24 hours at 2-8°C. Alternatively, aliquot and store at -20°C or -80°C for long term. Avoid repeated freeze-thaw cycles.

- **Tissue/Liver, Honey, Egg, Fodder:** Weigh 1 ± 0.05 g of homogenate and add to a 50 ml tube. Add 4 ml of deionized water, 0.5 mL of Solution 4, 100 µL of derivatization reagent and mix for 5 min. Incubate overnight at 37℃ or incubate with water bath at 50℃ for 3 hours. Add 5 mL of Solution 3, 0.4 mL of Solution 5 and 5 mL of ethyl acetate, oscillate for 5 min. Centrifuge at 4000 RPM for 10 min at room temperature. Collect 2.5 ml of supernatant and dry at 50-60°C with nitrogen or air. Dissolve the residue with 1 ml of n-hexane. Add 1 ml of Re-dissolve solution and mix fully by shaking for 30 sec. Centrifuge at 4000 RPM for 5 min at room temperature. Remove the hexane upper phase and take 50 µl of the lower phase for analysis.

- **Milk**: Centrifuge milk at 4000 RPM for 10 min at 15°C, and discard the upper fat layer. Take 5 ml of fat-free milk into a 50 ml tube, add 250 µl of Potassium nitroprusside solution (Solution 1) and mix for 30 sec. Add 250 µl of Zinc sulfate solution (Solution 2) and mix for 30 sec. Centrifuge at 4000 RPM for 10 min at room temperature. Collect 1.1 ml of supernatant to another centrifuge tube, add 4 ml of deionized water, 0.5 mL of 1 M HCl solution (Solution 4) and 100 µL of derivatization reagent, and mix for 5 min. Centrifuge at 4000 r/min at room temperature for 10 min. Transfer 2.5 mL of the upper liquid into another tube, blow-dry in nitrogen or air. Dissolve the residue with 1 mL n-hexane, add 1 mL of re-dissolve buffer and mix for 30 sec. Centrifuge at 4000 r/min at room temperature for 10 min. Discard the upper n-hexane, take 50 µL lower liquid to analyze
  Note: Sample dilution factor: 2, minimum detection dose: 0.1 ppb.

- **Milk/Egg Powder:** Weigh 1 ± 0.05 g milk powder into a tube and dissolve with 4 ml deionized water. Add 0.5 ml of 1M HCl solution and 100 µL of derivatization reagent, oscillate for 5 min. Incubate overnight at 37℃ or incubate with water bath at 50℃ for 3 hours. Add 250 µL of Solution 1, oscillate for 30s, then add 250 µL of Solution 2, mix for 30 sec. Centrifuge at 4000 r/min at 15℃ for 10 min. Transfer the supernatant to another tube, add 5mL of Solution 3, 0.4 mL of Solution 5 and 5 mL of ethyl acetate, mix for 5 min. Centrifuge at 4000 r/min at room temperature for 10 min. Take 2.5 mL of upper liquid to another tube, blow-dry in nitrogen or air. Dissolve the residue with 1mL n-hexane, add 1 mL of re-dissolve buffer and mix for 30 sec. Centrifuge at 4000 r/min at room temperature for 10 min. Discard the upper n-hexane, take 50 µL of lower liquid to analyze.

- **Meat sample:** Weigh 1±0.05 g of sample into 50 mL EP tube, add 4.5 mL of methyl alcohol and 0.5 mL of deionized water, mix for 2 min, centrifuge at 4000 r/min at room temperature for 5 min. Discard the liquid. Add 5 mL of acetonitrile and 5 mL of n-hexane, oscillate for 2 min, centrifuge at 4000 r/min at room temperature for 5 min. Discard the liquid. Add 4 mL of deionized water, 0.5 mL of solution 4 and 100 µL of derivatization reagent, oscillate for 5 min. Incubate overnight at 37℃ or incubate with water bath at 50℃ for 3 hours. Add 5 mL of Solution 3, 0.4 mL of Solution 5 and 5 mL of ethyl acetate, oscillate for 5 min. Centrifuge at 4000 r/min at room temperature for 10 min. Transfer 2.5 mL of supernatant to another tube, blow-dry in nitrogen or air. Dissolve the residue with 1 mL n-hexane, add 1 mL of 1×re-dissolve solution and mix for 30 sec. Centrifuge at 4000 r/min at room temperature for 10 min. Discard the upper n-hexane, take 50 µL of lower liquid to analyze.

**Note:**

» Fresh samples or recently obtained samples are recommended to prevent degradation and denaturalization that may lead to erroneous results. It is recommended to store samples to be used within 5 days at 4°C, within 1 month at -20°C and within 2 months at -80°C.

» Samples should be clear and transparent. Samples must be diluted so that the expected concentration falls within the kit's range.

» Please bring sample slowly to room temperature. Sample hemolysis will influence the result. Hemolyzed specimen should not be used. Samples that contain $NaN_3$ cannot be detected as it interferes with HRP.

» Always use non-pyrogenic, endotoxin-free tubes for blood collection.

**Sample dilution guideline:**

Estimate the concentration of the target in the sample and select the correct dilution factor to make the diluted target concentration fall near the middle of the kit's range. Generally, for high concentration (10000 pg/ml - 100000 pg/ml), dilute 1:100, for medium concentration (1000 pg/ml - 10000 pg/ml), dilute 1:10 and for low concentration (15.625 pg/ml - 1000 pg/ml), dilute 1:2. Very low concentrations (≤ 15.625 pg/ml ) do not need dilution. Dilute the sample with the provided Sample Diluent Buffer and mix thoroughly. Several trials may be necessary to determine the optimal dilution factor.

**3. Wash buffer**

Dilute the concentrated Wash buffer 20-fold (1/20) with distilled water (i.e. add 40 ml of concentrated wash buffer into 760 ml of distilled water).

**B. Assay Procedure**

Bring all reagents to room temperature prior to use.

1. Set standard, test sample and control (zero) wells on the pre-coated plate and record their positions. It is recommended to measure each standard and sample in duplicate.
2. Add 50 µl of the prepared standards solutions into the standard wells.
3. Add 50 µl of PBS into the control (zero) well.
4. Add 50 µl of appropriately diluted sample into test sample wells.
5. Immediately add 50 µl of HRP Conjugate to each well, then add 50 µL of antibody working solution, solution into each well. Add the solution at the bottom of each well without touching the side wall.
6. Cover the plate with the plate sealer. Tap the plate gently to ensure thorough mixing. Incubate at 25°C for 45 minutes.
7. Remove the cover, and wash the plate 5 times. Discard the solution without touching the side walls. Blot the plate on an absorbent material. Add 250 µl of wash buffer to each well and soak for at least 1 min. Discard the contents and clap the plate on absorbent filter papers or other absorbent material. Repeat this procedure for a total of five times.

**Note:** For automated washing, discard the solution in all wells and wash five times overfilling the wells with Wash buffer. After the final wash invert the plate and tap on absorbent filter papers or other absorbent material. It is recommended that the washer be set for a soaking time of 1 min.

8. Add 50 µl of substrate solution A and 50 µl of substrate solution B into each well. Cover the plate and incubate at 25°C in dark conditions for 15-20 minutes (incubation time is for reference only, do not exceed 30 minutes). When an apparent gradient appears in the standard wells the reaction can be terminated.
9. Add 50 µl of Stop solution into each well (including the blank well). There should be a colour change to yellow. Gently mix the plate to ensure thorough mixing.
10. Ensure that there are no fingerprints or water on the bottom of the plate, and that the fluid in the wells is free of bubbles. Measure the absorbance immediately. It is recommended to use a dual wavelength of 450/630 nm.

This assay is competitive, therefore there is an inverse correlation between SEM concentration in the sample and the absorbance measured. Create a graph with the log of the standard concentration (y-axis) and absorbance measured (x-axis). Apply a best fit trendline through the standard points. Use this graph calculate sample concentrations based on their OD values. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

**Note:** If the samples measured were diluted, multiply the dilution factor by the concentrations from interpolation to obtain the concentration before dilution.

**C. Precautions**

1. Equilibrate all reagents to room temperature (18-25°C) prior to use and centrifuge the tubes briefly in case any contents are trapped in the lid.
2. For each step in the procedure, total dispensing time for addition of reagents to the assay plate should not exceed 10 minutes.
3. Wash buffer may crystallize and separate. If this happens, please warm the tube and mix gently to dissolve.
4. Avoid foaming or bubbles when mixing or reconstituting components.
5. It is recommended to assay all standards, controls and samples in duplicate or triplicate.
6. Do NOT let the plate dry out completely during the assay as this will inactivate the biological material on the plate.
7. Ensure plates are properly sealed or covered during incubation steps.
8. Complete removal of all solutions and buffers during wash steps is necessary for accurate measurement readings.
9. To avoid cross contamination do not reuse pipette tips and tubes.
10. Do not use components from a different kit or expired ones.
11. Substrate solution A and substrate solution B are easily contaminated; work under sterile conditions when handling these solutions. The substrate solutions should also be protected from light. Unreacted substrate should be colorless or very light yellow in appearance. Aspirate the dosage needed with sterilized tips and do not dump the residual solution back into the vial.