



Technical Manual

AHD (Nitrofurantoin) ELISA Kit

- **Catalogue Code: FSES0004**
- **Competitive ELISA Kit**
- **Research Use Only**

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1. Key features and Sample Types

Sensitivity:

0.02 ppb (ng/mL)

Assay Procedure:

25°C, 45 min~15 min

Detection Limit:

Muscle (livestock), Liver, Honey, Milk - 0.04 ppb;

Milk powder, Feed, Egg powder, Egg - 0.04 ppb;

Muscle (fish, shrimp) - 0.06 ppb

Cross Reactivity:

AHD - 100%, AOZ, SEM, AMOZ < 0.1%,

Sample Recovery rate:

Muscle, Liver - 85%±25%, Honey, Milk - 80%±20%

Milk powder, Feed, Egg powder, Egg - 85%±25%

Storage:

2-8°C for 6 months.

Expiry:

See Kit Label

2. Storage

Store the kit at 2~8°C. Do not freeze any test kit components.

Return any unused microwells to their original foil bag and reseal them together with the desiccant provided and further store at 2 - 8°C.

3. Test Principle

This kit uses a Competitive-ELISA method. It can detect Nitrofurantoin (AHD) in samples, such as muscle, honey, milk, etc. This kit is composed of ELISA Microtiter plate, HRP conjugate, antibody working solution, standard and other supplementary reagents. The microtiter plate in this kit has been pre-coated with coupled antigen. During the reaction, AHD in the samples or standard competes with coupled antigen on the solid phase supporter for sites of anti-AHD antibody. Then Horseradish Peroxidase (HRP) conjugate is added to each microtiter plate well, and substrate reagent is added for color development. There is a negative correlation between the OD value of samples and the concentration of AHD. The concentration of AHD in the samples can be calculated by comparing the OD of the samples to the standard curve.

4. Kit Contents

Each kit contains reagents for 96 assays including:

No.	Component	96-WellKit
1	ELISA Microtiter plate	96 wells
2	Standards	1 mL each (0 ppb, 0.02 ppb, 0.06 ppb, 0.18 ppb, 0.54 ppb, 1.62 ppb)
3	Derivatization Reagent	10 mL
4	HRP Conjugate	5.5 mL
5	Antibody Working Solution	5.5 mL
6	Substrate Reagent A	6 mL
7	Substrate Reagent B	6 mL
8	Stop Solution	6 mL
9	20xConcentrated Wash Buffer	40 mL
10	2xReconstitution Buffer	50 mL
11	Plate Sealer	3 pieces
12	Sealed Bag	1 piece
13	Manual	1 copy

Note: All reagent bottle caps must be tightened to prevent evaporation and microbial pollution.

Additional materials required:

Other materials required but not supplied

- **Instruments:** Microplate reader, Printer, Homogenizer, Vortex Mixer, Nitrogen evaporators, Water bath, Centrifuge, Graduated pipette, Balance (sensitivity 0.01 g).
- **Micropipette:** Single channel (20-200 μ L, 100-1000 μ L), Multichannel (30-300 μ L).
- **Reagents:** Ethyl acetate, N-hexane, NaOH, Concentrated HCl, $K_2HPO_4 \cdot 3H_2O$, $ZnSO_4 \cdot 7H_2O$, $Na_2Fe(CN)_5 NO \cdot 2H_2O$.

5. Experimental Preparation

Bring all reagents and samples to room temperature before use.

Open the microplate reader in advance (30 min), preheat the instrument, and set the testing parameters.

1. Sample pre-treatment Notice:

Experimental apparatus should be clean, and the pipette should be disposable to avoid cross-contamination during the experiment.

2. Solution preparation

Solution 1: 0.36 M $\text{Na}_2\text{Fe}(\text{CN})_5\text{NO}$ Solution (for milk, milk powder, egg powder sample)
Dissolve 10.7 g of $\text{Na}_2\text{Fe}(\text{CN})_5\text{NO} \cdot 2\text{H}_2\text{O}$ to 100 mL with deionized water.

Solution 2: 1.04 M ZnSO_4 Solution (for milk, milk powder, egg powder sample)
Dissolve 29.8 g of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ to 100 mL with deionized water.

Solution 3: 0.1 M K_2HPO_4 Solution
Dissolve 11.4 g of $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ to 500 mL with deionized water.

Solution 4: 1 M HCl Solution
Dilute 8.6 mL of **Concentrated HCl** to 100 mL with deionized water.

Solution 5: 1 M NaOH Solution
Dissolve 4 g of **NaOH** to 100 mL with deionized water.

Solution 6: Reconstitution Buffer
Dilute the **2×Reconstitution Buffer** with deionized water. (2× Reconstitution Buffer (V): Deionized water (V) = 1:1). The Reconstitution buffer can be store at 4°C for 1 month.

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

3. Sample pre-treatment procedure

Targets may be distributed unevenly, resulting in no detection. To avoid this, ensure to take sufficient samples when sampling.

3.1 Pre-treatment of milk sample:

1. Take 5 mL of milk into 50 mL centrifuge tube, add 250 μL of **0.36 M $\text{Na}_2\text{Fe}(\text{CN})_5\text{NO}$ Solution** (Solution 1) and vortex for 30s, then add 250 μL of **1.04 M ZnSO_4 Solution** (Solution 2) and vortex for 30s, centrifuge at 4000 r/min for 10min at 15°C. If a refrigerated centrifuge is not available, chill sample to approx 15°C prior to centrifugation.
2. Take 1.1 mL of supernatant, add 4 mL of deionized water, 0.5 mL of **1 M HCl Solution** (Solution 4) and 100 μL of **Derivatization Reagent**, vortex for 5 min.
3. Incubate overnight at 37°C (about 16 hours) or incubate in water bath at 50°C for 3 hours (the effect of stratification will be affected when more than 50°C).
4. Add 5 mL of **0.1 M K_2HPO_4 Solution** (Solution 3), 0.4 mL of **1 M NaOH Solution** (Solution 5) and 5 mL of **Ethyl acetate**, vortex for 5 min.
5. Centrifuge at 4000 r/min at room temperature for 10 min.
6. Take 2.5 mL of upper liquid to another tube, dry at 50-60°C with nitrogen evaporators or water bath.
7. Dissolve the residual with 1 mL **N-hexane**, add 1 mL of **Reconstitution Buffer** (Solution 6) and vortex for 30s. Centrifuge at 4000 r/min at room temperature for 10

min.

8. Discard the upper n-hexane, take 50 μ L lower liquid for analysis.

Note: Sample dilution factor: 2, detection limit: 0.04 ppb

3.2 Pre-treatment of milk powder, egg powder sample:

1. Weigh 1 ± 0.05 g of sample into 50 mL centrifuge tube, add 4 mL of deionized water, 0.5 mL of **1 M HCl Solution** (Solution 4) and 100 μ L of **Derivatization Reagent**, vortex for 5 min.
2. Incubate overnight at 37°C (about 16 hours) or incubate with water bath at 50°C for 3 hours (the effect of stratification will be affected when more than 50°C).
3. Add 250 μ L of **0.36 M Na₂Fe (CN)₅ NO Solution** (Solution 1), vortex for 30s, then add 250 μ L of **1.04 M ZnSO₄ Solution** (Solution 2), vortex for 30s, centrifuge at 4000 r/min at 15°C for 10 min. If a refrigerated centrifuge is not available, chill sample to approx 15°C prior to centrifugation.
4. Take the supernatant to another tube, add 5 mL of **0.1 M K₂HPO₄ Solution** (Solution 3), 0.4 mL of **1 M NaOH Solution** (Solution 5) and 5 mL of **Ethyl acetate**, vortex for 5 min.
5. Centrifuge at 4000 r/min at room temperature for 10 min.
6. Take 2.5 mL of upper liquid to another tube, dry at 50-60°C with nitrogen evaporators or water bath.
7. Dissolve the residual with 1 mL **N-hexane**, add 1 mL of **Reconstitution Buffer** (Solution 6) and vortex for 30s. Centrifuge at 4000 r/min at room temperature for 10 min.
8. Discard the upper n-hexane, take 50 μ L lower liquid for analysis.

Note: Sample dilution factor: 2, detection limit: 0.04 ppb

3.3 Pre-treatment of honey, muscle (livestock, fish, shrimp), liver, feed, egg sample:

1. Weigh 1 ± 0.05 g of homogenate sample into 50 mL centrifuge tube, add 4 mL of deionized water, 0.5 mL of **1 M HCl Solution** (Solution 4) and 100 μ L of **Derivatization Reagent**, vortex for 5min.
2. Incubate overnight at 37°C (about 16 hours) or incubate in water bath at 50°C for 3 hours (the effect of stratification will be affected when more than 50°C).
3. Add 5 mL of **0.1 M K₂HPO₄ Solution** (Solution 3), 0.4 mL of **1 M NaOH Solution** (Solution 5) and 5 mL of **Ethyl acetate**, vortex for 5 min.
4. Centrifuge at 4000 r/min at room temperature for 10 min.
5. Take 2.5 mL of upper liquid to another tube, dry at 50-60°C with nitrogen evaporators or water bath.
6. Dissolve the residual with 1 mL **N-hexane**, add 1 mL of **Reconstitution Buffer** (Solution 6) and vortex for 30s. Centrifuge at 4000 r/min at room temperature for 10 min.
7. Discard the upper n-hexane, take 50 μ L lower liquid for analysis

Note: Sample dilution factor: 2, detection limit: 0.1ppb

detection limit of fish, shrimp: 0.15 ppb

6. Assay Procedure

Bring all reagents and samples to room temperature (25°C) before use. All the reagents should be mixed thoroughly by gently swirling before pipetting. Avoid foaming. The unused ELISA Microtiter plate should be sealed as soon as possible and stored at 2~8°C.

1. **Number:** number the sample and standard in order (multiple well), and keep a record of standard wells and sample wells. **Standard and Samples must be tested in duplicate.**
2. **Add Sample:** add 50 µL of **Standard or Sample** per well, then add 50 µL of **HRP Conjugate** to each well, then add 50 µL of **Antibody Working Solution**, cover the plate with plate sealer, vortex for 5s and mix thoroughly, incubate at 25°C for 45 min in the dark.
3. **Wash:** uncover the sealer carefully, remove the liquid in each well. Immediately add 300 µL of **Wash Buffer** (Solution 7) to each well and wash. Repeat wash procedure for 5 times, 30s intervals/time. Invert the plate and pat it against absorbent paper (If bubbles exist in the wells, clean tips can be used to prick them).
4. **Colour Development:** add 50 µL of **Substrate Reagent A** to each well, and then add 50 µL of **Substrate Reagent B**. Gently vortex for 5s to mix thoroughly. Incubate at 25°C for 15 min in the dark (The reaction time can be extended according to the actual colour change).
5. **Stop Reaction:** add 50 µL of **Stop Solution** to each well, gently vortex for 5s.
6. **OD Measurement:** determine the optical density (OD value) of each well at 450 nm (reference wavelength 630 nm) with a microplate reader. This step should be finished in 10 min after stop reaction.

7. Data Analysis

1. Absorbance (%) = $A/A_0 \times 100\%$

A: Average absorbance of standard or sample

A₀: Average absorbance of 0 ppb Standard

2. Drawing and calculation of standard curve

Create a standard curve by plotting the absorbance percentage of each standard on the y-axis against the log concentration on the x-axis to draw a semi-logarithmic plot. Add average absorbance value of sample to standard curve to get corresponding concentration. **If samples have been diluted, the concentration calculated from the standard curve must be multiplied by the dilution factor.**

For this kit, it is more convenient to use professional analysis form for accurate and fast analysis on a large number of samples.

8. Notes

1. The overall OD value will be lower when reagents have not been brought to room temperature before use or room temperature is below 25°C.
2. If the wells turn dry during the washing procedure, it will lead to bad linear standard curve and poor repeatability. Operate the next step immediately after wash.
3. Mix thoroughly and wash the plate completely. The consistency of wash procedure can strongly affect the reproducibility of this ELISA kit.
4. ELISA Microtiter plate should be covered by plate sealer. Avoid the kit to strong light.
5. **Each reagent is optimized for use in the FSES0004. Do not substitute reagents from any other manufacturer into the test kit. Do not combine reagents from other FSES0004 with different lot numbers.**
6. Substrate Reagent should be abandoned if it turns blue colour. When OD value of standard (concentration: 0) < 0.5 unit (A450nm < 0.5), it indicates the reagents are deteriorated.
7. Stop solution is caustic, avoid contact with skin and eyes.
8. As the OD values of the standard curve may vary according to the conditions of the actual assay performance (e.g. operator, pipetting technique, washing technique or temperature effects), the operator should establish a standard curve for each test.
9. Even the same operator might get different results in two separate experiments. In order to get reproducible results, the operation of every step in the assay should be controlled.
10. If the samples are not indicated in the manual, a preliminary experiment to determine the validity of the kit is necessary.
11. The kit is used for rapid screening of actual samples. If the test result is positive, the instrument method such as HPLC, LC/MS, etc. can be used for quantitative confirmation.

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If you are not satisfied with the quality of our products and our technical team cannot resolve your problem, we will give you 100% of your money back.

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