8th Edition, revised in February, 2018

AHD (Nitrofurantoin) ELISA Kit

Catalog No: E-FS-E004

96T

This manual must be read attentively and completely before using this product.

If you have any problems, please contact our Technical Service Center for help.

Phone: 240-252-7368(USA) Fax: 240-252-7376(USA)
Email: techsupport@elabscience.com
Website: www.elabscience.com

Please kindly provide us the lot number (on the outside of the box) of the kit for more efficient service.

Test principle

This kit uses Competitive-ELISA as the 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.

Technical indicator

Sensitivity: 0.02 ppb (ng/mL)

Reaction mode: 25℃, 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%

Kits components

Item	Specifications
ELISA Microtiter plate	96 wells
Standard Liquid	1 mL each (0 ppb,0.02 ppb,0.06 ppb,0.18 ppb,0.54 ppb,1.62 ppb)
Derivatization Reagent	10 mL
HRP Conjugate	5.5 mL
Antibody Working Solution	5.5 mL
Substrate Reagent A	6 mL
Substrate Reagent B	6 mL
Stop Solution	6 mL
20×Concentrated Wash Buffer	40 mL
2×Reconstitution Buffer	50 mL
Plate Sealer	3 pieces
Sealed Bag	1 piece
Manual	1 copy

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

Other materials required but not supplied

Instruments: Microplate reader, Printer, Homogenizer, Oscillators, Nitrogen evaporators, Water bath, Centrifuge, Graduated pipette, Balance (sensibility 0.01 g).

Micropipette: Single channel (20-200 μL, 100-1000 μL), Multichannel (30-300 μL).

Reagents: Ethyl acetate, N-hexane, NaOH, Concentrated HCl, $K_2HPO_4•3H_2O$, $ZnSO_4•7H_2O$, $Na_2Fe(CN)_5NO•2H_2O$.

Experimental preparation

Restore 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 pretreatment 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 $Na_2Fe(CN)_5NO$ Solution *(for milk, milk powder, egg powder sample)*

   Dissolve 10.7 g of **$Na_2Fe(CN)_5NO•2H_2O$** 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 **$ZnSO_4•7H_2O$** to 100 mL with deionized water.

   Solution 3: 0.1 M K_2HPO_4 Solution

   Dissolve 11.4 g of **$K_2HPO_4•3H_2O$** 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℃ 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 pretreatment procedure

3.1 Pretreatment of milk sample:

(1) Take 5 mL of milk into 50 mL centrifuge tube, add 250 μL of **0.36 M $Na_2Fe(CN)_5NO$ Solution** (Solution 1) and oscillate for 30s, then add 250 μL of **1.04 M $ZnSO_4$ Solution** (Solution 2) and oscillate for 30s, centrifuge at 4000 r/min for 10min at 15℃. If a refrigerated centrifuge is not available, chill sample to approx 15℃ 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**, oscillate for 5 min.

(3) Incubate overnight at 37℃(about 16 hours) or incubate in water bath at 50℃ for 3 hours (the effect of stratification will be affect when more than 50℃).

(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**, oscillate 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℃ with nitrogen evaporators or water bath.

(7) Dissolve the residual with 1 mL **N-hexane**, add 1 mL of **Reconstitution Buffer** (Solution 6) and oscillate for 30s. Centrifuge at 4000 r/min at room temperature for 10 min.

(8) Discard the upper n-hexane, take 50 μL lower liquid for analyze

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

3.2 Pretreatment 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 μL of **Derivatization Reagent**, oscillate for 5 min.

(2) Incubate overnight at 37℃(about 16 hours) or incubate with water bath at 50℃ for 3 hours (the effect of stratification will be affect when more than 50℃).

(3) Add 250 μL of **0.36 M $Na_2Fe(CN)_5NO$ Solution** (Solution 1), oscillate for 30s, then add 250 μL of **1.04 M ZnSO4 Solution** (Solution 2), oscillate for 30s, centrifuge at 4000 r/min at 15℃ for 10 min. If a refrigerated centrifuge is not available, chill sample to approx 15℃ prior to centrifugation.

(3) Take the supernatant to another tube, 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**, oscillate 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℃ with nitrogen evaporators or water bath.

(6) Dissolve the residual with 1 mL **N-hexane**, add 1 mL of **Reconstitution Buffer** (Solution 6) and oscillate for 30s. Centrifuge at 4000 r/min at room temperature for 10 min.

(7) Discard the upper n-hexane, take 50 μL lower liquid for analyze

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

3.3 Pretreatment 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 μL of **Derivatization Reagent**, oscillate for 5min.

(2) Incubate overnight at 37℃(about 16 hours) or incubate in water bath at 50℃ for 3 hours (the effect of stratification will be affect when more than 50℃).

(3) 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**, oscillate 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℃ with nitrogen evaporators or water bath.

(6) Dissolve the residual with 1 mL **N-hexane**, add 1 mL of **Reconstitution Buffer** (Solution 6) and oscillate for 30s. Centrifuge at 4000 r/min at room temperature for 10 min.

(7) Discard the upper n-hexane, take 50 μL lower liquid for analyze

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

detection limit of fish, shrimp: 0.15 ppb

Assay procedure

Restore all reagents and samples to room temperature (25℃) 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℃.

1. **Number:** number the sample and standard in order (multiple well), and keep a record of standard wells and sample wells. **Standard and Samples need test 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, oscillate for 5s and mix thoroughly, incubate at 25℃ for 45 min in shading light.
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 thick clean absorbent paper (If bubbles exist in the wells, clean tips can be used to prick them).
4. **Color Development:** add 50 μL of **Substrate Reagent A** to each well, and then add 50 μL of **Substrate Reagent B**. Gently oscillate for 5s to mix thoroughly. Incubate at 25℃ for 15 min in shading light (The reaction time can be extended according to the actual color change).
5. **Stop Reaction:** add 50 μL of **Stop Solution** to each well, gently oscillate 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.

Result analysis:

1. **Absorbance (%)=A/A_0×100%**

   A: Average absorbance of standard or sample

   A_0: 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.

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℃.
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 E-FS-E004. Do not substitute reagents from any other manufacturer into the test kit. Do not combine reagents from other E-FS-E004 with different lot numbers.**
6. Substrate Reagent should be abandoned if it turns blue color. When OD value of standard (concentration: 0)＜0.5 unit (A450nm＜0.5), it indicates the reagent be 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.

Storage and expiry date

Store the kit at 2~8℃. 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℃.

Expiry date: expiration date is on the packing box.