

Importance of Nitrofurantoin Determination

Antibiotic residues in foods pose a serious threat to public health. The nitrofurans class of broad spectrum antibiotics (Nitrofurantoin, furazolidone, furaltadone and nitrofurazone) are commonly used in food producing animals. Their potential for harmful effects on human health, specifically carcinogenicity, has led to bans on their use in food producing animals in many countries including the US, Canada, and the EU. These countries have also imposed bans on all imported foods containing nitrofurans residues. The monitoring of water sources and food products, such as meat, for antibiotic residues is necessary to ascertain that these compounds are not misused and do not present a danger to human and animal health.

The detection of nitrofurans themselves has proven challenging, as the drugs are rapidly metabolized after ingestion. The protein bound metabolites which are formed, however, persist in edible tissue for a considerable amount of time after treatment. AHD (1-aminohydantoin), the metabolite moiety derived from Nitrofurantoin, is not degraded by common cooking techniques and can be released from tissue under mildly acidic conditions, making it ideal for monitoring and detection in edible tissue.

The Abraxis Nitrofurantoin ELISA allows the determination of 41 samples in duplicate determination. Only a few grams or milliliters of sample are required. Hydrolysis and derivatization (overnight), and a subsequent solvent extraction step are necessary prior to assaying. The ELISA analysis can then be performed in less than 1 hour.

Performance Data

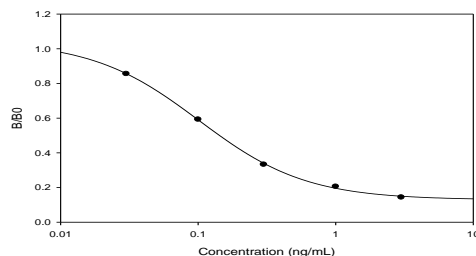
Test sensitivity: The limit of detection for Nitrofurantoin in various matrices are as follows:

Fish, shrimp, chicken, honey = 0.06 ppb
Milk = 0.24 ppb

Please contact Abraxis for additional extraction procedures.

Standard Curve: Determinations closer to the middle of the calibration range of the test yield the most accurate results.

These values are used for demonstration purposes only; do not use these values for your determinations.



Test reproducibility: Coefficients of variation (CVs) for standards: <10%; CVs for samples: <15%.

Selectivity: This ELISA recognizes Nitrofurantoin and not related compounds.

Cross-reactivities:

| | |
|----------------------|--------|
| Nitrofurantoin (AHD) | 100% |
| Furazolidone (AOZ) | <0.05% |
| Furaltadone (AMAZ) | <0.05% |
| Semicarbazide (SEM) | <0.05% |

Samples: To eliminate matrix effects in fish, shrimp, chicken, honey, and milk, a sample clean-up is required. See Preparation of Samples, section H.

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R062916

Nitrofurantoin (AHD) ELISA, Microtiter Plate

Enzyme-Linked Immunosorbent Assay for the Determination
of Nitrofurantoin in Contaminated Samples



Product No. 515666

1. General Description

The Nitrofurantoin ELISA is an immunoassay for the detection of the Nitrofurantoin metabolite (AHD). This test is suitable for the quantitative and/or qualitative detection of Nitrofurantoin in contaminated samples. Samples requiring regulatory action should be confirmed by HPLC, GC/MS, or other conventional methods.

2. Safety Instructions

The standard solutions in this test kit contain small amounts of AHD. In addition, the substrate solution contains tetramethylbenzidine and the stop solution contains diluted sulfuric acid. Avoid contact of stopping solution with skin and mucous membranes. If these reagents come in contact with skin, wash with water.

3. Storage and Stability

The Nitrofurantoin ELISA Kit should be stored in the refrigerator (4–8°C). The solutions must be allowed to reach room temperature (20–25°C) before use. Reagents may be used until the expiration date on the box.

4. Test Principle

The Abraxis Nitrofurantoin (AHD) Plate Kit applies the principles of enzyme linked immunosorbent assay (ELISA) to the determination of Nitrofurantoin. Calibrators (ready to use) and derivatized samples (please refer to reagent preparation section) are added, along with an AHD-HRP enzyme conjugate, to wells in a microtiter plate that contain immobilized antibodies specific for AHD. At this point, a competitive reaction occurs between the AHD which may be in the sample and the enzyme labeled AHD for the binding sites of the antibodies on the microtiter well. The reaction is allowed to continue for thirty (30) minutes. After a washing step, a substrate (Color Solution) is added.

The presence of AHD is detected by adding the "Color Solution," which contains the enzyme substrate (hydrogen peroxide) and the chromogen (3,3',5,5'-tetramethylbenzidine). The enzyme-labeled AHD bound to the AHD antibody catalyzes the conversion of the substrate/chromogen mixture to a colored product. After an incubation period, the reaction is stopped and stabilized by the addition of a diluted acid (Stopping Solution) and the color is evaluated using an ELISA plate reader. The concentrations of the samples are determined by interpolation using the standard curve constructed with each run. Since the labeled AHD (conjugate) was in competition with the unlabeled AHD (sample) for the antibody sites, **the color developed is inversely proportional to the concentration of AHD in the sample.**

5. Limitations of the Nitrofurantoin ELISA, Possible Test Interference

Numerous organic and inorganic compounds commonly found in samples have been tested and found not to interfere with this test. However, due to the high variability of compounds that might be found in samples, test interferences caused by matrix effects can not be completely excluded. Mistakes in handling the test can also cause errors. Possible sources for such errors include:

Inadequate storage conditions of the test kit, incorrect pipetting sequence or inaccurate volumes of the reagents, too long or too short incubation times during the immune and/or substrate reaction, extreme temperatures during the test performance (lower than 10°C or higher than 30°C).

The Abraxis Nitrofurantoin ELISA Kit provides screening results. As with any analytical technique (GC, HPLC, etc.), samples requiring regulatory action should be confirmed by an alternative method.

Working Instructions

A. Materials Provided

1. Microtiter plate coated with antibodies against AHD (12 X 8 strips).
2. Nitrofurantoin Standards/Calibrators (6): 0, 0.03, 0.1, 0.3, 1.0, and 3.0 ppb, 1.0 mL each. Standards are ready to use (already derivatized).
3. Nitrofurantoin-HRP Conjugate Solution, 6 mL.
4. Sample Treatment Buffer (10X) Concentrate, 50 mL (2 X 25 mL bottle). Use to dilute samples.
5. Wash Solution/Sample Diluent (10X) Concentrate, 50 mL.
6. Derivatization Reagent, 12 mL.
7. Color (Substrate) Solution (TMB), 12 mL.
8. Stop Solution, 12 mL.

B. Test Preparation

Micro-pipetting equipment and pipette tips for pipetting the standards and the samples are necessary. We recommend using a multi-channel pipette or a stepping pipette for adding the conjugate, substrate and stop solutions in order to equalize the incubation periods of the solutions on the entire microtiter plate. Please use only the reagents and standards from one package lot in one test, as they have been adjusted in combination.

1. Adjust the microtiter plate and the reagents to room temperature before use.
2. Remove the number of microtiter plate strips required from the foil bag. The remaining strips are stored in the foil bag and zip-locked closed. Store the remaining kit in the refrigerator (4-8°C).
3. The standard solutions, conjugate, substrate and stop solutions are ready to use and do not require any further dilutions.
4. Dilute the **Sample Treatment Buffer** concentrate at a ratio of 1:10. If using both bottles (50 mL), add to 450 mL of deionized or distilled water.
5. Dilute the **Wash Buffer/Sample Diluent** concentrate at a ratio of 1:10. If using the entire bottle (50 mL), add to 450 mL of deionized or distilled water.
6. The Derivatization Reagent is prepared in DMSO (Dimethylsulfoxide). It will freeze when stored at 4-8°C. The reagent bottle may be placed in a warm water bath to accelerate thawing.
7. The stop solution should be handled with care as it contains diluted H₂SO₄.

C. Assay Procedure

1. Add 100 µL of the **standard solutions and derivatized samples** into the wells of the test strips according to the working scheme given. Analysis in duplicate or triplicate is recommended.
2. Add 50 µL of **enzyme conjugate solution** to the individual wells successively using a multi-channel pipette or a stepping pipette. Cover the wells with parafilm or tape and mix the contents by moving the strip holder in a circular motion on the benchtop for 30 seconds. Be careful not to spill contents.
3. Incubate the strips for 30 minutes at room temperature.
4. After incubation, remove the covering and vigorously shake the contents of these wells into a sink. Wash the strips **four times** using the 1X washing buffer solution. Use at least a volume of 250 µL of washing buffer for each well and each washing step. Remaining buffer in the wells should be removed by patting the plate dry on a stack of paper towels.
5. Add 100 µL of **substrate (color) solution** to the wells. Cover the wells with parafilm or tape and mix the contents by moving the strip holder in a circular motion on the benchtop for 30 seconds. Incubate the strips for 20 minutes at room temperature. Protect the strips from direct sunlight.
6. Add 100 µL of **stop solution** to the wells in the same sequence as for the substrate solution.
7. Read the absorbance at 450 nm using a microplate ELISA photometer within 15 minutes after the addition of the stopping solution.

D. Evaluation

The evaluation of the ELISA can be performed using commercial ELISA evaluation programs 4-Parameter (preferred) or Logit/Log. For manual evaluation, calculate the mean absorbance value for each of the standards. Calculate the %B/B₀ for each standard by dividing the mean absorbance value for each standard by the Zero Standard (Standard 0) mean absorbance. Construct a standard curve by plotting the %B/B₀ for each standard on the vertical linear (y) axis versus the corresponding Nitrofurantoin concentration on the horizontal logarithmic (x) axis on graph paper. %B/B₀ for samples will then yield levels in ppb of Nitrofurantoin by interpolation using the standard curve. Samples showing lower concentrations of Nitrofurantoin compared to Standard 1 (0.03 ng/mL) should be reported as containing < 0.06 ppb for fish, shrimp, chicken, and honey, and < 0.24 ppb for milk. Samples showing a higher concentration than Standard 6 (3 ng/mL) must be diluted further to obtain accurate results.

E. Additional Materials (not included with the test kit)

1. Micro-pipettes with disposable plastic tips (10-200 and 200-1000 µL)
2. Multi-channel pipette (10-250 µL) or stepper pipette with plastic tips (10-250 µL)
3. Microtiter plate reader (wave length 450 nm)
4. Centrifuge, capable of spinning at 3,000 rpm
5. Vortex Mixer
6. 50 mL centrifuge tubes
7. 10 mL glass tubes or vials
8. Mixer (Stomacher, Ultraturax)
9. Incubator (37°C)
10. Water bath (80-100°C)
11. Nitrogen (suggested but not essential)
12. Timer
13. Tape or Parafilm

F. Additional Reagents (not included with the test kit)

1. Ethyl Acetate
2. n-Hexane
3. 1N HCl
4. 1 N NaOH
5. Distilled or deionized water

G. Working Scheme

The microtiter plate consists of 12 strips of 8 wells, which can be used individually for the test. The standards must be run with each test. Never use the values of standards which have been determined in a test performed previously.

Std 0-Std 6: Standards

0; 0.03; 0.1; 0.3; 1.0; 3.0 ppb

Samp1, Samp2, etc.: Samples

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|-------|--------|--------|---|---|---|---|---|---|----|----|----|
| A | Std 0 | Std 4 | Samp 2 | | | | | | | | | |
| B | Std 0 | Std 4 | Samp 2 | | | | | | | | | |
| C | Std 1 | Std 5 | etc. | | | | | | | | | |
| D | Std 1 | Std 5 | | | | | | | | | | |
| E | Std 2 | Std 6 | | | | | | | | | | |
| F | Std 2 | Std 6 | | | | | | | | | | |
| G | Std 3 | Samp 1 | | | | | | | | | | |
| H | Std 3 | Samp 1 | | | | | | | | | | |

H. Preparation of Samples (Derivatization and Extraction)

a) Fish/Shrimp/Chicken (0.06 ppb Sensitivity)

1. Weigh 1.0 g of homogenized fish or de-shelled shrimp or homogenized chicken sample (should have a paste-like consistency) into a 50 mL centrifuge tube.
2. Add 4.0 mL of distilled or deionized water, 0.5 mL 1N HCl, and 100 µL of Derivatization Reagent. Vortex thoroughly for 1 minute.
3. Incubate at 37°C overnight (approximately 16 hours).
4. Add 5.0 mL of the diluted **Sample Treatment Buffer**, 0.4 mL of 1N NaOH and 5.0 mL of ethyl acetate and vortex thoroughly for 1 minute.
5. Centrifuge tube for 10 minutes at 3000 rpm.
6. Transfer 2.0 mL of the ethyl acetate layer (top layer) into a clean vial or glass tube.
7. Evaporate to dryness at 40-60°C under a gentle stream of nitrogen.
8. Dissolve the residue with 1.0 mL of n-hexane, vortex. Add 0.8 mL of diluted **Wash Solution/Sample Diluent Solution**, and vortex thoroughly for 1 minute.
9. Boil the sample for approximately 3 minutes at 80-100°C.
10. Centrifuge vial/tube for 10 minutes at 3000 rpm.
11. The aqueous layer (lower layer) will then be analyzed as sample (Assay Procedure, step 1).

The ELISA result will be multiplied by a factor of 2 to obtain the final AHD concentration in the sample (the factor is necessary to account for the sample dilution in the procedure). Samples showing lower concentrations than standard 1 (0.03 ppb) should not be multiplied by the factor (2) but should be reported as containing <0.06 ppb. Highly contaminated samples (those outside of the calibration range of the assay) must be diluted and re-analyzed.

b) Milk (0.24 ppb Sensitivity)

1. Centrifuge 3-4 mL of milk at 3000 rpm for 10 minutes.
2. Carefully take 1.0 mL of the lower layer (**Must avoid taking up the upper fat layer**), then follow step H.a.2 to 10 of the **Fish/Shrimp/Chicken Derivatization and Extraction** procedure.
3. Take 100 µL of the lower layer and dilute with 300 µL of **Wash Solution/Sample Diluent Solution**.
4. Analyze as sample (Assay Procedure, step 1).

The ELISA result will be multiplied by a factor of 8 to obtain the final AHD concentration in the sample.

c) Honey (0.06 ppb Sensitivity)

1. Weigh 1.0 g of sample and follow steps H.a.2 and 3 listed in the **Fish/Shrimp/Chicken Derivatization and Extraction** procedure.
2. Add 5 mL of the diluted **Sample Treatment Buffer**, 0.5 mL of 1N NaOH and 5 mL of ethyl acetate and vortex thoroughly for 1 minute.
3. Follow steps H.a.5 to 11 listed in the **Fish/Shrimp/Chicken Derivatization and Extraction** procedure.

The ELISA result will be multiplied by a factor of 2 to obtain the final AHD concentration in the sample.

NOTE: An alternative derivatization (section H, procedure a, step 3, above) may be performed as follows: Incubate samples at 60°C for 3 hours. Proceed to step 4 above.

When using unknown samples of shrimp and fish with the incubation reduced from 16 hours at 37°C to 3 hours at 60°C, as described above, the recoveries were between 80-120% of those using the 16 hours at 37°C derivatization procedure. When using known **negative** samples of shrimp and fish spiked and derivatized with the incubation reduced from 16 hours at 37°C to 3 hours at 60°C, the recoveries were between 70-110%.