# Competitive Enzyme Immunoassay Kit for Quantitative Analysis of Furaltadone metabolite (AMOZ)

## 1. Background

Nitrofurans are synthetic broad-spectrum antibiotics, which are frequently employed in animal production for its excellent antibacterial and pharmacokinetic properties. They had been also used as growth promoters in pig, poultry and aquatic production. In long term studies with lab animals indicated that the parent drugs and their metabolites showed carcinogenic and mutagenic characteristics. This has led to a prohibition of nitrofurans for the treatment of animals used for food production. The nitrofuran drugs furaltadone, nitrofurantoin and nitrofurazone were banned from use in food animal production in the EU in 1993, and the use of furazolidone was prohibited in 1995.

The analysis of nitrofuran drugs residue needs to be based on the detection of the tissue bound metabolites of the nitrofuran parent drugs, since the parent drugs are very rapidly metabolized, and the tissue bound nitrofuran metabolites will retain for a long time, therefore the metabolites are used as the target in the detection of the abuse of nitrofurans. Furazolidone metabolite (AMOZ), Furaltadone metabolite (AMOZ), Nitrofurantoin metabolite (AHD) and Nitrofurazone metabolite (SEM).

AMOZ-residues are determined most commonly by LC-MS or LC-MS/MS. Enzyme immunoassays, compared with chromatographic methods, show considerable advantages regarding sensitivity, detection limit, technical equipment and time requirement.

## 2. Test Principle

This ELISA kit is designed to detect AMOZ based on the principle of indirect-competitive enzyme immunoassay. The microtiter wells are coated with capture BSA-linked antigen. AMOZ in sample competes with the antigen coated on the microtiter plate for the antibody added. After the addition of enzyme conjugate, chromogenic substrate is used and the signal is measured by a spectrophotometer. The absorption is inversely proportional to AMOZ concentration in the sample.

## 3. Applications

This kit can be used in quantitative and qualitative analysis of AMOZ residue in aquatic products (fish and shrimp), etc.

## 4. Cross-reactions

Furaltadone metabolite(AMOZ)……………………100%
Furazolidone metabolite(AMOZ)……………………<0.1%
Nitrofurantoin metabolite(AHD)……………………<0.1%
Nitrofurazone metabolite(SEM)……………………<0.1%
Furaltadone……………………………………11.1%
Furazolidone……………………………………<0.1%
Nitrofurantoin……………………………………<1%
Nitrofurazone……………………………………<1%

## 5. Materials Required

### 5.1 Equipments

----Microtiter plate spectrophotometer (450nm/630nm)
----Rotary evaporator or nitrogen drying instruments
----Homogenizer
----Shaker
----Vortex mixer
----Centrifuge
----Analytical balance (inductance: 0.01g)
----Graduated pipette: 10ml
----Rubber pipette bulb
----Volumetric flask: 100ml.
----Glass flask: 10ml
----Polystyrene centrifuge tube: 2ml, 10ml, 50ml
----Micropipettes: 20ul-200ul, 100ul-1000ul, 250ul-multipipette

### 5.2 Reagents

-----Ethyl acetate (AR)
-----Methanol (AR)
-----n-hexane ( or n-heptane ) (AR)
-----Dipotassium hydrogen phosphate trihydrate ($K_2HPO_4.3H_2O$) (AR)
-----Concentrated hydrochloric acid
-----Sodium hydroxide (NaOH, AR)

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----Deionized water

## 6. Kit Components

- Microtiter plate with 96 wells coated with antigen
- Standard solutions(6 bottles)
  **0ppb, 0.05ppb,0.15ppb,0.45ppb,1.35ppb,4.05ppb**
- Spiking standard solution: (1ml/bottle) …...**100ppb**
- Enzyme conjugate 1ml…………..…………….red cap
- Antibody solution 7ml ………..……………..green cap
- solution A 7ml…………………………………white cap
- solution B 7ml……………………………..…… red cap
- stop solution 7ml ……………………………yellow cap
- 20×concentrated wash solution 40ml
  ……………………………………..…transparent cap
- 2×concentrated extraction solution 50ml
  ………………………………………………..….blue cap
- 2-Nitrobenzaldehyde 15.1mg……………..….white cap

## 7. Reagents Preparation

**Solution 1**: Derivative reagent:

Add methanol to the bottle with 2-Nitrobenzaldehyde and diluted to 10ml. (at the concentration of 10mM).

**Solution 2**: 0.1M dipotassium hydrogen phosphate solution ($K_2HPO_4.3H_2O$):

Weigh 22.8g of dipotassium hydrogen phosphate trihydrate($K_2HPO_4.3H_2O$) and dissolved with deionized water to 1L.

**Solution 3**: 1M hydrochloric acid solution:

Transfer 8.3ml of concentrated hydrochloric acid and dilute to 100ml with deionized water.

**Solution 4**: 1M sodium hydroxide solution:

Weigh 4.0g of sodium hydroxide and dissolve with deionized water and dilute to 100ml.

**Solution 5**: Extraction solution

Dilute 2×concentrated extraction solution with deionized water in the volume ratio of 1:1(*e.g. 10ml of 2×concentrated extraction solution + 10ml of deionized water*). This solution can be conserved for 1month at 4℃.

**Solution** 6: Wash solution:

Dilute the 20×concentrated wash solution with deionized water in the volume ration of 1:19(*e.g. 10ml of 20×concentrated wash solution + 190ml of deionized water*), which will be used to wash the plates. This diluted solution can be conserved for 1 month at 4℃.

## 8. Sample Preparations

### 8.1 Notice and precautions before operation

*(a) Please use one-off tips in the process of experiment, and change the tips when absorbing different reagent.*
*(b) Make sure that all experimental instruments are clean.*
*(c) the derivative reagent can be conserved at 2-8℃ for half a year;*
*(d) Potassium phosphate dibasic solution can be conserved at 2-8℃ for three months;*
*(e) The hydrochloric acid solution can be conserved at room temperature for 3 months;*
*(f) The sodium hydroxide solution can be conserved for 3 months at room temperature;*
*(g) Keep untreated samples in freeze;*
*(h) Treated samples can be conserved for 24h at 2-8℃ in darkness .*

### 8.2 fish and shrimp samples

-----Homogenize the samples with homogenizer;

------Weigh 1.0±0.05g of the homogenate into a 50ml polystyrene centrifuge tube, add 4ml of deionized water, 0.5ml of 1M hydrochloric acid (**solution 3**) and 100µl of derivative reagent (**solution 1**), shake completely for 2min;

-----Incubate at 37℃ overnight ( about 16h) or incubate at 50℃ for 3 hours (get out and shake for 30s when 1.5 hours;

------Add 5ml of 0.1M dipotassium hydrogen phosphate solution (**solution 2**), 0.4ml of 1M sodium hydroxide solution (**solution 4**) and 5ml of ethyl acetate, shake fiercely for 30s;

-----Centrifuge for separation: at least 3000g / 10min / at ambient temperature;

-----Take 2.5ml of the supernatant organic phase into a 10ml clean glass tube, dry with 50℃ water bath under nitrogen gas flow;

-----Dissolve the dry leftover with 1ml of n-hexane (or n-heptane), add 1ml of extraction solution (**solution 5**) , mix completely.

-----Centrifuge for separation: at least 3000g / 10min / at ambient temperature;

-----Remove the supernatant organic phase, take 50µl of the substrate aqueous phase for assay.

***Dilution factor of samples:*** *2.*

## 9. Assay process

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### 9.1 Notice before assay:

*9.1.1 Make sure all reagents and microwells are all at room temperature (20-25℃).*

*9.1.2 Return all the rest reagents to 2-8℃ immediately after used.*

*9.1.3 Washing the microwells correctly is an important step in the process of assay; it is the vital factor to the reproducibility of the ELISA analysis.*

*9.1.4 Avoid the light and cover the microwells during incubation.*

### 9.2 Assay Steps:

9.2.1 Take all reagents out at room temperature (20-25℃) for more than 30min, homogenize before use.

9.2.2 Get the microwells needed out and return the rest into the zip-lock bag at 2-8℃ immediately.

9.2.3 The concentrated wash solution and concentrated extraction solution should be rewarmed to be at room temperature before use.

9.2.4 **Number:** Numbered every microwell positions and all standards and samples should be run in duplicate. Record the standards and samples positions.

9.2.5 **Add standard solution**: Add 50μl of standard solution(**kit components**) or prepared sample to corresponding wells.

9.2.6 **Mix the enzyme conjugate and antibody solution**: Mix the concentrated enzyme conjugate with antibody solution in the volume ratio of 1:10(e.g. *0.5ml of concentrated enzyme conjugate + 5ml of antibody solution*), mix completely.

9.2.7 **Add the mixture of concentrated enzyme conjugate and antibody solution**: Add 50μl of the *mixture of concentrated enzyme conjugate and antibody solution*. Mix gently by rocking the plate manually and incubate for 30min at 25℃ with cover.

9.2.6 **Wash**: Remove the cover gently and pour the liquid out of the wells and rinse the microwells with 250μl diluted wash solution (**solution 6**) at interval of 10s for 4-5 times. Absorb the residual water with absorbent paper (*the rest air bubble can be eliminated with unused tip*).

9.2.8 **Coloration**: Add 50μl of solution A(**kit components**) and 50μl of solution B(**kit components**) to each well. Mix gently by rocking the plate manually and incubate for 15min at 25℃ with cover(see 12.8).

9.2.9 **Measure**: Add 50μl of the stop solution(**kit components**) to each well. Mix gently by rocking the plate manually and measure the absorbance at 450nm against an air blank (It's suggested measure with the dual-wavelength of 450/630nm. Read the result within 5min after addition of stop solution. We can also measure by sight without stop solution in short of the ELISA reader)

## 10. Results

### 10.1 Percentage absorbance

The mean values of the absorbance values obtained for the standards and the samples are divided by the absorbance value of the first standard (zero standard ) and multiplied by 100%. The zero standard is thus made equal to 100% and the absorbance values are quoted in percentages.

$$\text{Absorbance}(\%) = \frac{B}{B_0} \times 100\%$$

B ——absorbance standard (or sample)

$B_0$ ——absorbance zero standard

### 10.2 Standard Curve

To draw a standard curve: Take the absorbance value of standards as y-axis, semi logarithmic of the concentration of the AMOZ standard solution (ppb) as x-axis.

--- The AMOZ concentration of each sample (ppb), which can be read from the calibration curve, is multiplied by the corresponding dilution factor of each sample followed, and the actual concentration of sample is obtained.

**Please notice:**

Special software has been developed for all data analysis, which can be provided on request.

## 11. Sensitivity, accuracy and precision

**Sensitivity**: **0.05ppb**

**Detection limit**

Aquatic products(fish and shrimp)………………… 0.1ppb

**Accuracy**

Aquatic products(fish and shrimp)……….………… 95±25%

**Precision:** CV of the ELISA kit is less than 10%.

## 12. Notice

*12.1 The mean values of the absorbance values obtained for the standards and the samples will be reduced if the reagents and samples have not been regulated to room*

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*temperature ( 20-25℃).*

*12.2 Do not allow microwells to dry between steps to avoid unsuccessful reproducibility and operate the next step immediately after tap the microwells holder.*

*12.3. Homogenize each reagent before using.*

*12.4. Keep your skin away from the stop solution for it is the 2M $H_2SO_4$ solution.*

*12.5 Don't use the kits out of date. Don't exchange the reagents of different batches, or else it will drop the sensitivity.*

*12.6 Keep the ELISA kits at 2-8℃,do not freeze. Seal rest microwell plates, Avoid straight sunlight during all incubations. Covering the microtiter plates is recommended.*

*12.7 Substrate solution should be abandoned if it turns colors. The reagents may be deteriorated if the absorbance value (450/630nm) of the zero standard is less than 0.5 (A450nm<0.5).*

*12.8 The coloration reaction needs 10-15min after the addition of solution A and solution B; But you can prolong the incubation time to 20min or more if the color is too light to be determined., never exceed 30min,On the contrary, shorten the incubation time properly.*

*12.9 The optimal reaction temperature is 25℃. Higher or lower temperature will lead to the changes of sensitivity and absorbance values.*

**13. Storage condition and storage period**

Storage condition: 2-8℃.

Storage period: 12months

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