Competitive Enzyme Immunoassay Kit for Quantitative Analysis of Nitrofurantoin Metabolite (AHD)

1. Background

Nitrofurans are synthetic broad-spectrum antibiotics, which are frequently employed in animal production for the antibacterial and pharmacokinetic properties. They had been also used as growth promoters in swine poultry and aquatic production. Studies with experimental animals the parent drugs and their metabolites indicated that these drugs are carcinogenic and mutagenic. 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 Nitrofurantoin was prohibited in 1995.

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, e.g. furazolidone metabolite (AOZ), furaltadone metabolite (AMOZ), Nitrofurantoin metabolite (AHD) and nitrofurazone metabolite (SEM).

AHD are determined most commonly by LC-MS, or LC-MS/MS. Enzyme immunoassays, compared with chromatography methods, shows great advantages regarding sensitivity, detection limit, technical equipment and time requirement.

2. Test Principle

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

3. Applications

This kit is used for the quantitative and qualitative analysis of AHD in honey.

4. Cross-reactions

Nitrofurantoin metabolite (AHD)……100%

Furazolidone metabolite (AOZ)……...<0.1%

Furaltadone metabolite (AMOZ)……<0.1%

Nitrofurazone metabolite (SEM)……..<0.1%

Furazolidone……...…<1%

Furaltadone……<1%

Nitrofurantoin……...…13%

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, 1L

----Glass test tube: 10ml

----Polystyrene centrifuge tube: 2ml,50ml

----Micropipettes: 20ul-200ul,
100ul-1000ul, 250ul-multipipette

5.2 Reagents

----Ethyl acetate (AR)

----n-Hexane (or normal heptane) (AR)

----Dipotassium hydrogen phosphate trihydrate ($K_2HPO_4.3H_2O$) (AR)

----Methanol (AR)

----Concentrated hydrochloric acid (HCl, AR)

----Sodium hydroxide (NaOH, AR)

----Deionized water

6. Kit Components

- Microtiter plate with 96 wells coated with antigen
- AHD standard solutions. (1ml×6 bottles)
  0ppb, 0.025ppb, 0.075ppb, 0.225ppb, 0.675ppb, 2.025ppb
- Spiking standard solution: 1ml, **100ppb**
- Concentrated enzyme conjugate 1ml…..……..red cap
- Enzyme conjugate dilution solution 10ml…green cap
- Solution A 7ml..……..……..…..white cap
- Solution B 7ml……..……..…red cap
- Stop solution 7ml..……yellow cap
- 20×concentrated wash solution 40ml...transparent cap

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- 2×Concentrated extraction solution 50ml……. blue cap
- 2-nitrobenzaldehyde 15.1mg ……black cap

7. Reagents Preparation:

Solution 1: derivative reagent:

Add methanol to the bottle with 2-nitrobenzaldehyde and diluted to 10ml. (the final concentration is 10mM)

Solution 2: 0.1M K_2HPO_4:

Dissolve 22.8g $K_2HPO_4.3H_2O$ with deionized water, dilute to 1L.

Solution 3: 1M HCl solution:

Dilute the concentrated hydrochloric acid 8.3ml with deionized water, dilute to 100ml.

Solution 4: 1M NaOH:

Weight 4g sodium hydroxide (NaOH) dissolved with deionized water, dilute to 100ml.

Solution 5: Extraction solution:

Dilute the 2×extraction concentrate solution with deionized water in the volume ratio of 1:1, which will be used for sample extraction. The diluted extraction solution can be conserved for a month at 4℃.

Solution 6: Wash solution:

Dilute the 20×wash concentrated solution with deionized water in the volume ratio of 1:19, which will be used to rinse the plates. This diluted wash solution can be conserved for a month at 4℃.

8. Sample Preparation

8.1 Notice and precautions before operation

(a) Please use one-off tips in the process of experiment, and change the tips if different reagent is absorbed.

(b) Make sure that all experimental tools are clean.

(c) Derivative reagent can be stored for half a year at 2-8℃.

(d) Store the untreated sample in freeze.

(e) The treated sample can be stored for 24 hours, 2-8℃, in darkness.

8.2 Honey

----Weigh 1.0±0.05g honey sample into a 50ml polystyrene centrifuge tube;

----Add 4ml deionized water, shake to dissolve;

----Add 0.5ml 1M HCl (**solution** 3) and derivative reagent (**solution** 1) 100ul and shake completely for 2 min with shaker.

----Incubation at 37 ℃ overnight (approx. 16 hours).

----Add 0.1M K_2HPO_4 solution (**solution** 2) 5ml, 1M NaOH (**solution** 4) 0.4ml and ethyl acetate 5ml,shake completely for 30s with shaker.

----Centrifuge: 10min, at least 3000g, at 20-25℃.

----Transfer the supernatant ethyl acetate layer 2.5ml into a dry glass centrifuge tube(10ml) and dry at 50-60℃ with water bath nitrogen gas stream.

----Dissolve the leftover with 1ml n-hexane (or n-heptane) and vortex for 30s, and then add 1ml extraction solution (**solution** 5) and vortex for 1min. Avoid emulsification during vortex, and then transfer to 2ml polystyrene tube.

----Centrifuge for 10min, at least 3000g, at 20-25℃.

----Remove the supernatant phase, and use 50ul per well of the substrate layer for assay.

9. Assay process

9.1 Notice before assay:

9.1.1 Make sure all reagents and microwells are all at room temperature (20-25℃) for more than 30min until rewarm.

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

9.1.3 Wash 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 process:

9.2.1 Take all reagents out at room temperature (20-25℃) for more than 30min, shake gently 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 diluted wash solution should be brought to 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/ sample:** add 50 µl of each standard solution or prepared sample to the corresponding wells.

9.2.6 **Premix**: mix the enzyme conjugate dilution solution and concentrated enzyme conjugate in the volume ratio of 10:1, shake gently. **Notice: the enzyme conjugate mixture can not be conserved, please use it immediately.**

9.2.7 Add enzyme conjugate mixture: Add 50µl antibody-enzyme mixture. Mix gently by shaking the plate manually and incubate for 30min at 25℃ with cover.

9.2.7 **Wash**: Remove the cover gently and pour the liquid out of the wells and wash the microwells with 250µl diluted wash solution (**solution** 6) at intervals of 10s. Tap the microwells holder upside down vigorously against

absorbent paper to ensure complete removal of liquid from the wells (repeat 4-5 times).

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

9.2.9 **Measure**: Add 50µl the stop solution to each well. Mix gently by rocking the plate manually and measure the absorbance at 450nm (It's suggested measure with the dual-wavelength of 450/630nm. Read the result within 5min after addition of stop solution)

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.

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

B ——absorbance of standard (or sample)

B_0 ——absorbance of zero standard

10.2 Standard Curve

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

----The AHD concentration of each sample (ppb), which can be read from the calibration curve, is multiplied by the corresponding dilution rate 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.

Dilution factor of samples: 2.

11. Sensitivity, accuracy and precision

Test Sensitivity: 0.025ppb

Detection limit……0.05ppb

Accuracy……100±30%

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 temperature (20-25℃).

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

12.3 Shake each reagent gently before use.

12.4 Keep your skin away from the stop solution for it is the 0.5M 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. Avoid straight sunlight during all incubations. Covering the microtiter plates is recommended.

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

12.8 The coloration reaction need 10-15min after the addition of solution A and solution B; But you can prolong the incubation time ranges to 20min if the color is too light to be determined, never exceed 25min. 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: 12 months