Competitive Enzyme Immunoassay Kit for Quantitative Analysis of Semicarbazide (SEM)

1. Background

Nitrofurans are synthetic broad-spectrum antibiotics, which are frequently employed in animal production for its excellent antibacterial and pharmacokinetic properties. Long term research indicates that nitrofurans and their metabolites lead to caner and gene mutations in lab animals, thus these drugs are being prohibited in therapy and feedstuff.

Nitrofurans are metabolized inside the body very fast, and their metabolites combined with the tissues would exist for quite a long time, so residue analysis of these drugs will depend on the detection of their metabolites, including furazolidone metabolite (AOZ), furaltadone metabolite (AMOZ), nitrofurantoin metabolite (AHD) and nitrofurazone metabolite (SEM).

The common approach to detect nitrofurazone metabolite is LC-MS and LC-MS/MS. The ELISA test, in which specific antibody of SEM derivative is used is more accurate, sensitive, and simple to operate. The assay time of this kit is only 1.5h.

2. Test Principle

This kit is designed to detect semicarbazide (SEM) based on the principle of “indirect-competitive” enzyme immunoassay. The microtiter wells are coated with capture BSA-linked antigen. SEM 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 SEM concentration in the sample.

3. Applications

This kit can be used for the quantitative and qualitative analysis of SEM in honey.

4. Cross-reactions

Semicarbazide……100%
Nitrofurazone……25%
Furazolidone metabolite(AOZ)……<0.1%
Furaltadone metabolite(AMOZ)……<0.1%
Nitrofurantoin metabolite(AHD)……<0.1%
Furazolidone……<1%
Furaltadone……<1%
Nitrofurantoin……<1%

5. Materials Required

5.1 Equipments:

----Microtiter plate spectrophotometer (450nm/630nm)
----Rotary evaporator or nitrogen drying instruments
----Homogenizer (or stomacher)
----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)
----Potassium phosphate dibasic 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
- SEM standard solutions×6 bottles: (1ml/bottle)
0ppb, 0.1ppb, 0.3ppb, 0.9ppb, 2.7ppb, 8.1ppb
- Spiking standard solution: (1ml/bottle) **100ppb**
- Enzyme conjugate 12ml ……red cap
- Antibody solution 7ml ……green cap
- Substrate Solution A 7ml ……white cap
- Substrate 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……whitecap

7. Reagents Preparation:

Solution 1: Derivative reagent: Add methanol to the bottle with 2-nitrobenzaldehyde and dilute to 10ml. (the final concentration is 10mM)

Solution 2: 0.1M K_2HPO_4:

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

Solution 3: 1M HCl solution:

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

Solution 4: 1M Sodium hydroxide (NaOH):

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

Solution 5: extraction solution

Dilute the 2×concentrated extraction 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×concentrated wash solution with deionized water in the volume ratio of 1:19, which will be used to rinse the plate. This solution can be conserved at 4℃ for 1 month (the wash solution is only for this kit, do not use with other kit)

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 when absorbing different reagent.

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

(c) The derivative reagent can be conserved at 2-8℃ for a year;

(d) Keep untreated samples in freeze;

(e) Treated samples can be stored for 24h at 2-8℃ in darkness.

(f) K_2HPO_4 solution can be stored at 2-8℃ for three months;

(g) The HCl solution can be stored at room temperature for 3 months;

(h) The NaOH solution can be stored for 3 months at room temperature;

8.2 Honey sample

----Weigh 1.0±0.05g of the sample into a 50ml polystyrene tube;

----Add 4ml deionized water, vortex to dissolve completely,

----Add 0.5ml 1M HCl solution (**solution** 3) and 100μl derivative reagent (**solution** 1), and shake completely with shaker;

----Incubate at 37 ℃ over night (about 16h):

----Then add 5ml 0.1M K_2HPO_4 (**solution** 2), 0.4ml 1M NaOH solution (**solution** 4) and 5ml ethyl acetate; shake fiercely for 30s with shaker;

----Centrifuge at room temperature (20-25 ℃) for 10min, at least 3000g;

----Take 2.5ml of the supernatant organic phase into a 10ml clean glass test tube, dry with 50-60℃ nitrogen gas flow;

----Dissolve the dry leftover with 1ml n-hexane (or normal heptane), vortex for 30s, then add 1 ml extraction solution (**solution** 5), vortex for 1min to mix completely

----Centrifuge at room temperature (20-25 ℃) for 10min, at least 3000g;

----Remove the supernatant organic phase and take 50μl of the substrate water phase 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℃).

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, 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/sample and antibody solution:** add standard solution or sample solution 50μl to the corresponding well, then add antibody solution 50μl to each well, mix gently, incubate for 30min **at 4℃** in darkness 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 5 times. Absorb the residual water with absorbent paper.

9.2.7 **Add enzyme conjugate**: add enzyme conjugate 100µ to each well, mix gently, then incubate for 30min at 25℃in darkness with cover, take out and repeat the wash step;

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

9.2.9 **Measure**: add 50µl of the stop solution to each well. Mix gently by shaking 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 then 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 (0ppb)

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 SEM standards solution (ppb) as x-axis.

----The SEM 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:

Software has been developed for data analysis, which can be provided upon request.

Dilution factor of samples……..2

11. Sensitivity, accuracy and precision

Test Sensitivity: **0.1ppb**

Detection limit……..……0.1ppb

Accuracy:……90±20%

Precision:

Variation coefficient 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 reproducibility and operate the next step immediately after tap the microwells holder.

12.3. Shake each reagent gently before using.

12.4. Keep your skin away from the stop solution for it is 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. 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 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 needs 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 25min,On the contrary, shorten the incubation time properly.

12.9 The optimal reaction temperature of adding SEM standard solution, antibody solution is 4℃. And after adding enzyme conjugate and Solution A, Solution B, 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.