Competitive Enzyme Immunoassay for Quantitative Analysis of Nitroimidazoles

1. Background

Nitroimidazoles are broad-spectrum anthelminthics, which can kill amebic protozoa and anaerobic bacillus, as well as cocci. They can also be used for stopping surgical site infections. Overdose nitroimidazoles in food would cause anorexia, naupathia, diarrhea and other side effects to human, and strict MRLs have been set against them.

This kit is a new generation product for drug residue detection based on ELISA technology. It is fast, simple, accurate and sensitive. And it requires only 2.0 hours in one operation, which considerably minimizes work intensity and operation error. It can be used for nitroimidazoles residue detection in honey.

2. Test Principle

This ELISA kit is designed to detect nitroimidazoles based on "indirect-competitive" enzyme immunoassay. The microtiter wells are coated with BSA-linked nitromidazole antigen. Nitroimidazoles in the sample competes with the precoated antigen for binding to the limited number of antibody. After the addition of enzyme conjugate and TMB substrate, the signal is measured with an ELISA photometer. The absorption is inversely proportional to the nitroimidazoles concentration in the sample, compared with the standard curve, nitroimidazoles residue in the sample can be calculated.

3. Applications

This kit can be used for rapid test of nitroimidazoles in honey.

Please notice: orange honey and buckwheat honey are not applicable with this ELISA kit.

4. Cross reactions

Metronidazole.....100%
Secnidazole.....182%
Ornidazole.....103%
Tinidazole.....<1%
Dimetridazole.....<1%
Ronidazole..... <1%
MNZOH..... <1%

5. Equipment needed but not provided

5.1 Equipments

----ELISA reader (450nm/630nm)
----Rotary evaporator / N2 drying instrument
----Shaker
----Vortex mixer
----Centrifuge
----Analytical balance (inductance: 0.01g)
----Graduated pipette: 10ml
----Rubber pipette bulb
----Volumetric flask: 100ml, 500ml;
----Glass test tube: 10 ml;
----Polystyrene centrifuge tube: 50ml, 2ml
----Micropipettes: 20μl-200μl, 100μl-1000μl
300μl-multipipette

5.2 Reagents

----Ethyl Acetate (AR)
----n-hexane (AR)
----Disodium phosphate dodecahydrate ($Na_2HPO_4{\cdot}12H_2O$, AR)
----Sodium dihydrogen phosphate dihydrate ($NaH_2PO_4{\cdot}2H_2O$, AR)
----Sodium carbonate anhydrous(Na_2CO_3, AR)
----Sodium bicarbonate($NaHCO_3$, AR)
----Deionized water

6. Kit Components

- Microtiter plate with 96 wells coated with antigen
- Standard solutions(5 bottles×1ml/bottle)
  0ppb, 0.05ppb, 0.15ppb, 0.45ppb, 1.35ppb
- 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
- Extraction solution 50ml.....blue cap

7. Reagents Preparation

Solution 1: 0.2M phosphas buffer (pH=6)

Weigh 4.4g $Na_2HPO_4{\cdot}12H_2O$ and 13.68g $NaH_2PO_4{\cdot}2H_2O$,, dissolve with deionized water and dilute to 500ml;

Solution 2: Wash solution

Dilute the 20X concentrated wash solution with deionized water in the volume ratio of 1:19, which will be used to rinse the plates. The diluted wash solution can be conserved for one 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) Treated samples can be stored at 2-8°C for 24h in dark.

8.2 Honey

------Weigh 2.0±0.05g honey into a 50ml polystyrene centrifuge tube;

------Add 2ml 0.2M phosphas buffer solution (**solution** 1);

------Add 8ml ethyl acetate, shake for 5min via shaker till all samples dissolved , centrifuge for 5min, at least 3000g at room temperature (20-25℃);

------Transfer 4ml supernatant organic phase to 10ml clean glass tube, dry under 50-60℃ water bath nitrogen gas;

------Add 1ml n-hexane, vortex for 30s, add 0.5ml extraction solution (kit component), vortex for 30s, centrifuge for 5min, at least 3000g at room temperature (20-25℃);

------Remove the supernatant organic phase, and take 50µl of the substrate solution 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 ℃). **Notice: The antibody solution should be stored at 4℃, which will be used immediately after taking out.**

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 Please avoid direct sunlight during the incubation, which means the plate should be covered with the plate cover provided in the kit.

9.2 Assay Steps

9.2.1 Take all reagents out at room temperature (20-25℃) for more than 30min. Shake gently before use. **Notice: The antibody solution should be stored at 4℃, which will be used immediately after taking out.**

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

9.2.5 **Add standard /sample and antibody**: Add 50µl of standard solution or prepared sample to corresponding wells. Add 50µl antibody solution(**The antibody solution should be stored at 4 ℃, which will be used immediately after taking out**), mix gently by shaking the plate manually and incubate for 60min at **4**℃ with cover (or in dark place).

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** 2) 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).

9.2.7 **Add enzyme conjugate**: Add 100µl enzyme conjugate to each well, mix gently by shaking the plate manually and incubate for 30min at 25℃ with cover. Take out and **wash the plate again following 9.2.6**

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 15 min at 25℃ with cover(**see 12.8**).

9.2.9 **Measure**: Add 50µl 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 from the standards and the samples are divided by the absorbance value of the first standard (zero standard) and multiplied by 100%.

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

B ——the mean absorbance value of each standards or each samples

B_0 ——absorbance value of zero standard

10.2 Standard Curve

---To draw a standard curve, the absorbance value of standards as y-axis, semilogarithmic of the concentration

of the standards (ppb) as x-axis.

---The nitroimidazoles 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 reduction, which can be provided on request.

Sample dilution factor:

Honey.....0.5

11. Sensitivity, accuracy and precision

Test Sensitivity: **0.05ppb**

Detection limit

Honey.....0.1ppb

Accuracy

Honey.....100±15%

Precision

C.V. 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℃). **The antibody solution should be stored at 4℃, which will be used immediately after taking out. If the antibody solution is return to room temperature before assay, the OD values will be higher, and the result of the assay will not be right.**

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 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. 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 need 30min after the addition of solution A and solution B, but you can prolong the incubation time ranges to 35min or more if the color is too light to be determined, never exceed 40min, on the contrary, shorten the incubation time properly.

12.9 After adding standard, antibody solution, the incubation temperature is **0-4**℃. While after adding enzyme conjugate, substrate A and B, the incubation temperature will be **25**℃. Please make sure the temperature is correct during all steps. Higher or lower temperature will lead to experiment failure.

13. Storage

Storage condition: 2-8℃.

Storage period: 12months