Competitive Enzyme Immunoassay Kit for Quantitative Analysis of Benzyl penicillin

1. Background

Benzyl penicillin (BP) is a broadly applied antibiotic, which once played very important role in preventing and curing animal diseases. For it causes anaphylactic reaction and resistance, in EU, US and China, it is being restricted. The common instrumental analysis of this drug is limited because of the complicated operation and high expense, while this kit is a new product based on ELISA technology, which is fast, easy, accurate and sensitive compared with common instrumental analysis and only needs 1.5 hours in one detection, so it can considerably minimize operation error and work intensity.

2. Test Principle

This kit is based on indirect-competitive ELISA technology. The microtiter wells are coated with coupling antigen. BP residue in the sample competes with the antigen coated on the microtiter plate for the antibody. After the addition of enzyme labeled anti-antibody, TMB substrate is used to show the color. Absorbance of the sample is negatively related to the BP reside in it, after comparing with the Standard Curve, multiplied by the dilution factor, BP residue quantity in the sample can be calculated.

3. Applications

This kit can be used in quantitative and qualitative analysis of BP residue in honey and milk, milk powder, ice cream and butter.

4. Cross-reactions

Benzyl penicillin (BP)..... 100%
Ampicillin 0.7%
Cloxacillin 0.2%
Dicloxacillin 0.1%
Amoxicillin .....<0.1%
Ceftiofur .....<0.1%

5. Materials Required

5.1 Equipments

----Microtiter plate spectrophotometer (450nm/630nm)
----Rotary evaporator or nitrogen gas drying system
----Homogenizer
----Shaker
----Vortex mixer
----Centrifuge
----Analytical balance (inductance: 0.01g)
----Graduated pipette: 10ml
----Rubber pipette bulb
----Polystyrene centrifuge tubes: 50ml
----Glass test tube: 10ml
----Volumetric flask: 100ml, 500ml
----Micropipettes:20ul-200ul,200ul-10000ul,
250ul-multipipette

5.2 Reagents

----Sodium hydroxide(NaOH, AR)
----Acetonitrile (AR)
----N-hexane(AR)
----Concentrated sulfuric acid (H_2SO_4, for honey)
----Concentrated hydrochloric acid (HCl, for honey)
----Methanol (AR)
----Deionized water

6. Kit Components

- Microtiter plate with 96 wells coated with antigen
- Penicillin standard solutions. (1ml×6 bottles)
  0 ppb, 0.1ppb, 0.3ppb, 0.9ppb, 2.7ppb, 8.1ppb
- Spiking standard control: 1ml, **1ppm**
- Enzyme conjugate (12ml).....red cap
- Antibody solution (7ml) .....green cap
- Solution A (7ml) .....white cap
- Solution B (7ml) .....red cap
- Stop solution (7ml) .....yellow cap
- 20×concentrated washsolution (40ml)..transparent cap
- 2×Concentrated extraction solution (50ml).....blue cap

7. Reagents Preparation

Solution 1: Acidic acetonitrile solution (for honey sample)
Take 100ml acetonitrile and mix with 150μl 2M sulfuric acid;
Solution 2: 1M HCl (for honey)
Dilute 41.5ml concentrated HCl with deionized water, and dilute to 500ml;
Solution 3: 1M NaOH (for honey)
Dissolve 4.0 g sodium hydroxide with deionized water and

dilute to 100ml;

Solution 4: Extraction solution

Dilute 2×concentrated extraction solution with deionized water in the volume ration of 1:1, which will be used for sample extraction. This solution can be stored for 1 month at 4℃.

Solution 5: Wash solution

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

8. Sample Preparations

8.1 Notice and precautions for 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.

8.2 Honey sample

----Weigh 4.0±0.05g honey sample into a centrifuge tube, then add 0.5ml 1M NaOH (solution 3), shake with shaker , then keep steady for 20min;

----Add 0.5ml 1M HCl (solution 2), shake with shaker (its pH is about 3, if not, please regulate with HCl or NaOH), then add 7ml acidified acetonitrile solution (the pH is about 4.0, see solution 1), shake for 10min with shaker, then centrifuge at room temperature (20-25℃) for 10min,at least 3000g;

----Take 3ml of the supernatant into a 10ml clean glass tube, dry with 50-60℃ water bath nitrogen gas flow;

----Dissolve the dry leftover with 1ml extraction solution (solution 4), vortex for 1min until it is completely dissolved;

----Take 50 μl of the prepared solution for assay.

8.3 Milk sample

----Take 20μl fresh milk into a 2ml centrifuge tube.

----Add 380μl extraction solution (solution 4), mix for 1min to completely mixed;

----Take 50 μl of the prepared solution for assay.

8.4 Milk powder sample

----Weigh 1.0±0.05g milk powder sample.

----Dissolve with 5ml deionized water, mix completely

----Transfer 50μl to 350μl extraction solution(solution 4), vortex for 30s to mix it completely;

----Take 50 μl of the prepared solution for assay.

8.5 Ice cream and butter

----Weigh 1.0±0.05g ice cream or butter sample into a 50ml polystyrene centrifuge tube,

----Add 1ml methanol, mix to dissolve completely, then add 4ml extraction solution (solution 4), mix completely with shaker.

----Dilute the above solution with extraction solution at the volume ration 1:1(eg. 200μl sample solution + 200ul extraction solution), mix completely.

----Take 50 μl of the prepared 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℃).

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 diluted wash 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/sample**: Add 50 μl of standard solution or prepared sample to corresponding wells. Add 50μl antibody solution. Mix gently by rocking the plate manually and incubate for 30min at 37℃ 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 7) 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 solution:** Add enzyme conjugate 100μl to each well, mix gently by rocking the plate manually and incubate for 30min at 37℃ with cover.

Repeat the wash step again.

9.2.8 **Coloration**: Add 50μl solution A and 50μl solution B to each well. Mix gently by rocking the plate manually and incubate for 15min at 37℃ 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.

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

B ——absorbance standard (or sample)

B0 ——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 BP standards solution (ppb) as x-axis.

--- The BP 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 interpretation, which can be provided on request.

Dilution factor of samples:

Milk……20

Honey……1

Milk powder……40

Ice cream and butter……10

11. Sensitivity, accuracy and precision

Test Sensitivity: **0.1ppb**

Detection limit:

Honey sample……0.1ppb

Milk sample……2ppb

Milk powder sample……4ppb

Ice cream/butter…… 1ppb

Accuracy:

Honey …… 70±10%

Milk / milk powder / ice cream / butter…… 85±25%

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 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 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 10-15min after the addition of solution A and solution B; But you can prolong the incubation time ranges from 20min to 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 is 37℃. Higher or lower temperature will lead to the changes of sensitivity and absorbance values.

13. Storage

Storage condition: 2-8℃.

Storage period: 12 months