Competitive Enzyme Immunoassay Kit for Quantitative Analysis of Ampicillin

1. Background

Ampicillin belongs to the beta-lactams family, 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 1.5h in single 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. Ampicillin 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 ampicillin reside in it, after comparing with the Standard Curve, multiplied by the dilution factor, ampicillin residue quantity in the sample can be calculated.

3. Applications

This kit can be used in quantitative and qualitative analysis of ampicillin residue in animal tissue (muscle, liver, aquatic products), honey and milk, etc.

4. Cross-reactions

Ampicillin …… 100%
Penicillin……..52%
Penicillin V……<20%

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: 2ml, 4ml, 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)
----Sodium nitroprusside dihydrate ($Na_2[Fe(CN)_5NO].2H_2O$, AR, for milk)
----Zinc sulfate ($ZnSO_4.7H_2O$, AR, for milk)
----Concentrated sulfuric acid (H_2SO_4, for honey)
----Concentrated hydrochloric acid (HCl, for honey)
----Deionized water

6. Kit Components

- Microtiter plate with 96 wells coated with antigen
- Penicillin standard solutions. (1ml×6 bottles) **0 ppb, 0.2ppb, 0.6ppb, 1..8ppb, 5.4ppb, 16.2ppb**
- Spiking standard solution: 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×Wash concentrate solution (40ml)…..transparent cap
- 2×Concentrated extraction solution (50ml)…..blue cap

7. Reagents Preparation

Solution 1: 0.1M NaOH solution
Dissolve 0.4g of Sodium hydroxide e with deionized water and dilute to 100ml;

Solution 2: Acetonitrile-0.1M NaOH (for tissue)
Take 84ml of acetonitrile and mix with 16ml of 0.1M NaOH.

Solution 3: **Solution C**: 0.36M sodium nitroprusside (for milk)

Dissolve 10.7g of sodium nitroprusside dihydrate with water and dilute to 100ml.

Solution 4: Solution D: 1M zinc sulfate (for milk)

Dissolve 28.8g of $ZnSO_4.7H_2O$ with water and dilute to 100ml.

Solution 5: 2M H2SO4

Dilute 10ml of the concentrated sulfuric acid with deionized water to 100ml.

Solution 6: Acidic acetonitrile solution (for honey sample)

Take 100ml of acetonitrile and mix with 150μl of 2M sulfuric acid;

Solution 7: 1M HCl (for honey)

Dilute 8.3ml of concentrated HCl with deionize d water, and dilute to 100ml;

Solution 8: 1M NaOH (for honey)

Dissolve 4.0g of sodium hydroxide with deionized water and dilute to 100ml;

Solution 9: 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 10: 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.

(c) For method B of milk sample, If milk is still muddy after centrifuge, please add Solution C and D again and repeat the centrifuge step.

(d) Keep collected sample in freeze.

(e) Prepared sample should be used for assay immediately.

8.2 Tissue (chicken, duck, pork, liver, fish and shrimp)

----Homogenize the samples with homogenizer;

----Weigh 2.0±0.05g of the homogenate into a 50ml polystyrene centrifuge tube, add 8ml of acetonitrile-0.1M NaOH (**solution** 2), vortex for 2min, then centrifuge at room temperature (20-25℃) for 5min, at 3000g;

----Take out 1ml of the supernate into a 10ml clean glass test tube, dry with 50-60℃ water bath nitrogen gas flow;

----Dissolve the dry leftover with 1ml of n-hexane, then add 1ml of extraction solution (**solution** 9), vortex for 1min to mix thoroughly, centrifuge at room temperature (20-25℃) for 5min, at 3000g;

----Remove the supernatant n-hexane phase, take 50μl of the substrate aqueous phase and dilute with 200μl of extraction solution (**solution** 9), mix thoroughly.

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

Dilution factor: 20

8.3 Honey

----Weigh 4.0±0.05g of honey into a centrifuge tube, then add 0.5ml of 1M NaOH (**solution** 8), shake thoroughly with shaker for 5min, then keep steady for 20min ;

----Add 0.5ml of 1M HCl (**solution** 7), shake with shaker for 5min (pH is about 3, if not, please regulate with HCl or NaOH), then add 7ml of acidified acetonitrile solution (the pH is about 4.0, **solution** 6), shake for 5min, then centrifuge at room temperature (20-25℃) for 5min,at 3000g;

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

----Dissolve the dry leftover with 1ml extraction solution (**solution** 9), vortex for 1min;

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

Dilution factor: 1

8.4 Milk sample

8.4.1 Method A

----Take 1ml fresh milk into a 4ml centrifuge tube, centrifuge at room temperature (20-25℃) for 5min,at 3000g, and then remove the upper fat layer.

----Take 20μl of the substrate layer and mix with 780μl of extraction solution (**solution** 9), mix completely;

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

Dilution factor: 40

8.4.2 Method B

----Take 2ml of fresh milk into a 10ml centrifuge tube.

----Add 50μl of Solution C (**solution** 3), mix completely, and then add 50μl of Solution D (**solution** 4), vortex for 1min, then centrifuge at room temperature (20-25℃) for 5min,at

3000g.

----Take the supernatant liquid and diluted it with extraction solution in the ratio of 1:19. (Such as take 20μl of the supernate, and mix with 380μl extraction solution (**solution** 9), mix completely;

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

Dilution factor: 20

8.4.3 Method C

----Take 4ml of fresh milk into a 10ml centrifuge tube., centrifuge at room temperature (20-25℃) for 5min,at 3000g, remove the fat ;

---- Take 2ml of the fat removed milk into a 4ml centrifuge tube, add 8ml of acetonitrile-0.1M NaOH (**solution** 2), shake with shaker for 5min, and then centrifuge at room temperature (20-25℃) for 5min,at 3000g;

----Take 1ml of the supernate into a 10ml clean glass tube, dry with 50-60℃ water bath nitrogen gas flow;

----Dissolve the dry leftover with 1ml of n-hexane, and then add 1ml of extraction solution (**solution** 9), vortex for 5min to mix, centrifuge at room temperature (20-25℃) for 5min,at 3000g.

---- Remove the supernatant n-hexane phase, and take 50μl of the substrate aqueous layer and mix with 150μl of extraction solution (**solution** 9).

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

Dilution factor: 20

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 and antibody solution**: Add 50 μl of standard solution(**Kit component**) or prepared sample to corresponding wells. Add 50μl antibody solution(**Kit component**). 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 of diluted wash solution (**solution** 10) 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 100μl of enzyme conjugate(**Kit component**) to each well, mix gently by rocking the plate manually and incubate for 30min at 25℃ with cover. Repeat the ***wash step*** again.

9.2.8 **Coloration**: Add 50μl of solution A(**Kit component**) and 50μl of solution B(**Kit component**) 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 component**) 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)

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 ampicillin standards solution (ppb) as x-axis.

--- The ampicillin 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.

11. Sensitivity, accuracy and precision

Test Sensitivity: **0.2ppb**

Detection limit:

Tissue……4ppb

Honey……0.5ppb

Milk:

Method A……8ppb

Method B & C……4ppb

Accuracy:

Tissue…… 90±20%

Honey ……90±20%

Milk…… 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 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 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. And you can prolong the incubation time 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 25℃. Higher or lower temperature will lead to the changes of sensitivity and absorbance values.

13. Storage

Storage condition: 2-8℃

Storage period: 12 months