Competitive Enzyme Immunoassay Kit for

Quantitative Analysis of Clenbuterol (Type II)

1.Background

Clenbuterol is a kind of ß-agonist, which is illegally added in feeds for boosting the lean meat percentage of the high fat animals and promoting the animal growth. Because its additive dosage in feed is 5~10 times more than therapy dosage, it resides more inside the animal body, which bring great harm to the consumers.

This kit is a new product based on ELISA technology, which is fast, easy, accurate and sensitive compared with common instrumental analysis, so it can considerably minimize operation error and work intensity. This kit has been documented in Doc. 558 of Ministry of Agriculture(China, PRC).

2. Test Principle

This kit is based on indirect-competitive ELISA technology. The microtiter wells are coated with coupling antigen. Clenbuterol 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 Clenbuterol reside in it, after comparing with the Standard Curve, multiplied by the dilution multiple, Clenbuterol residue quantity in the sample can be calculated.

Time cost : only 50min.

3. Applications

This kit can be used in quantitative and qualitative analysis of Clenbuterol residue in animal tissue (muscle and liver),urine and feed.

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4. Cross-reactions

Clenbuterol……100%

Salbutamol……13%

terbutaline……<1%

Fenoterol ……<1%

Mabuterol……<1%

Cimaterol……<1%

Bromobuterol……<1%

Salmeterol……<1%

isoprenaline iso-prenalinum ……<5%

5. Materials Required

5.1 Equipments:

- Microtiter plate spectrophotometer (450nm/630nm)

- Rotary Evaporator/Nitrogen drying instrument

- Homogenizer or stamocher

- Shaker

- Gyroscope or vortex mixer

- Centrifuge

- Analytical balance (inductance: 0.01g)

- Graduated pipette: 10ml

- Rubber pipette bulb

- Volumetric flask:100mlC500mlC1L

- Glass test tube:10ml

- Polystyrene Centrifuge tube:2mlC10mlC50ml

- Micropipettes:20ml~200mlC100ml~1000ml

- 250ul-multipipette

- RIDA C18 column

5.2 Reagents:

- Methanol (AR)(For tissue sample)

- Sodium hydrate(AR)

- Sodium Chloride(AR)

- n-hexane(AR)

- concentrated hydrochloric acid

- Potassium dihydrogen phosphate (AR) (For tissue sample)

- Tris Base(For tissue sample)

- deionized water

6. Kit Components

1.Microtiter plate with 96 wells coated with coupling antigen

2.Standard solutions(6 bottles×1ml/bottle)

0ppb,**0.1ppb**,**0.3ppb**,**0.9ppb**,**2.7ppb**,**8.1ppb**

3.High concentration standard solution: (1ml/bottle) **100ppb**

4.Enzyme-labeled secondary antibody solution 7ml … red cap

5. Antibody work solution 10ml …….green cap

6. Solution A 7ml..... white cap

7. Solution B 7ml.....red cap

8. Stop solution 7ml…….yellow cap

9. 20×concentrated wash solution 40ml…transparent cap

7. Reagents Preparation

solution 1:50mM hydrochloric acid(For meat and liver samples low in fat)

Dilute 4.17ml concentrated hydrochloric acid with deionized water to 1L.

solution 2:pH3.0 50mM potassium dihydrogen phosphate buffer solution(For sample columniation)

weigh 3.40g potassium dihydrogen phosphate, dilute to 500ml with deionized water.(regulate its pH with phosphoric acid or sodium hydrate to 3.0)

solution 3:pH3.0 500mM potassium dihydrogen phosphate buffer solution

(For meat and liver samples low in fat)

Weigh 34.02g potassium dihydrogen phosphate, dilute with deionized water to 500ml(regulate its pH with phosphoric acid or sodium hydrate to3.0).

solution 4:pH8.5 50mM Tris Base buffer solution.

(For meat and liver samples rich in fat)

Weigh 3.03g Tris Base, dissolved with deionized water and dilute to 500ml(regulate its pH to 8.5 with hydrochloric acid)

solution 5:1M hydrochloric acid(for feed sample)

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

solution 6:1M sodium hydrate solution

(For meat, liver and feed samples low in fat)

Dissolve 4.0g sodium hydrate with 100ml deionized water and dilute to 100ml.

solution7 : 0.1M sodium hydrate solution(for tissue not filtered with column)

Weigh 0.83ml sodium hydrate, dissolve with deionized water and dilute to 100ml.

Solution 8: 4% NaCl solution

Weigh 4.0g sodium chloride, dissolve with deionized water to 100ml.

solution 9: 4%NaCl- 0.1M HCl- methanol mixed solution

Take 70ml 4% NaCl solution, 20ml 0.1M hydrochloric acid solution and 10ml methanol, mix them together completely. Solution 10 : wash solution

Dilute the concentrated wash solution with deionized water in the volume ratio of 1:19(or according to the requirement), which will be used to rinse the plate. The diluted wash solution can be conserved for a month at 4C.

8. Sample Preparations

8.1 Notice and precautions for the users before operation:

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

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

(c) Tissue sample solution extracted with Method (a) can be reserved at 4C for 3 days.

The recovery rate of tissue sample solution extracted with Method (b) (has sodium hydrate in it)will decrease by 10% if being kept for over 4h, it should be detected immediately and it can not be conserved if sodium hydrate is added.

(d) Treated feed sample can not be stored, it must be detected immediately.

(e) Samples homogenized with hydrochloric acid should keep steady at 2-8C for 3 days.

(f) Samples extracted potassium dihydrogen phosphate buffer solution should keep steady at 2-8C for 2 days before purified with RIDA C18 column, and remember to homogenize before use.

(g) methanol extracted sample purified with RIDA C18 column can be store in freeze for 2 months, and keep steady for 2 weeks at 2-8C.

(h) the dry leftover dissolved with deionized water should be keep steady at 2-8C for 1week.

8.2 Urine

Take 20ml transparent urine sample for assay(please centrifuge (at least 3000g, 15C for 10min until it is transparent) or filter if the urine sample is muddy), and keep the unused samples in freeze.

8.3 Tissues(meat and liver sample) low in fat.

Method (a)

----Weigh 5.0±0.05g comminuted tissue sample into a 50ml polystyrene centrifuge tube, and add 25ml 50mM hydrochloric acid(see solution 1), shake for 1.5h with shaker to homogenize.

----Weigh 6.0±0.05g of the homogenate(equal to 1g sample) into a 50ml polystyrene centrifuge tube.

----Centrifuge, at least 3000g, for 15min at 10-15C.

----Transfer the supernatant transparent liquid to another 50ml clean polystyrene centrifuge tube, then add 300ml 1M sodium hydrate(see solution 6), shake for 15min.

----Add 4ml pH3.0 500mM potassium dihydrogen phosphate buffer solution(see solution 3) ,mix simply, and then keep at 4C for at least 1.5h or over night**(IMPORTANT)**

---- Centrifuge at least 3000g, at 10-15C for 15min, and separate the supernate (it should be transparent, **IMPORTANT**), and warm it to room temperature(20-25C); and then purified with RIDA C18column(See RIDA C18 purification operation).

Method (b)

---Weigh 2±0.05g homogenized sample into a 50ml polystyrene centrifuge tube,

---- add 6ml 4%NaCL-0.1M HCl,- methanol solution(see solution 9) shake with shaker to mix completely.

----Keep steady for 10min, centrifuge at least 3000g for 5min.

Liver sample: take 1ml supernate , add 20µl 1M NaOH(See solution 6) ,and mix completely,(its pH is about 8 after mix)

Muscle sample: take 1ml supernate ,add 30µl 1M NaOH (See solution 6) ,and mix completely,(pH is about 8 after mix)

----Take 20ml of the prepared solution for assay

8.4 Meat and tissues rich in fat

----Weigh 5.0±0.05 g comminuted sample into a 50ml polystyrene centrifuge tube, and mix with 25ml pH8.5 50mM Tris buffer solution(see solution 4), shake for 0.5h with shaker to homogenize.

----Add15ml n-hexane, shake for 5min with shaker to eliminate the fat.

----Centrifuge at least 3000g, at 10-15C for 15min.

----Remove the supernatant n-hexane phase and thin fat phase in the middle-level with transferpettor.

----Add 15ml n-hexane, and shake for 5min with shaker to eliminate the fat.

----Add 0.5ml concentrated hydrochloric acid, and then shake for 1h;

----Weigh 6.0g±0.05 g of the homogenate (equal to 1.0g tissue sample) into a 10ml polystyrene centrifuge tube.

----The following steps will repeat steps from **8.3 Method(a) Step 2,** and then purify with RIDA C18 column follow the next methods(Remember to strictly control the flow speed of the liquid in the column)

RIDA C18 purification steps:

----Wash the column with 3ml methanol, and the speed is 1drop/s.

----Wash the column with 2ml wash solution (pH3.0 50mM potassium dihydrogen phosphate buffer solution, see solution 2).

----Transfer the sample into the column(all the supernate of the tissue sample, 15 drops/min).

----Wash the column with 2ml wash solution (pH3.0 50mM potassium dihydrogen phosphate buffer solution, see solution 2).

----Remove the residual liquid with positive pressure, and blow with weak air or nitrogen gas for 2min to dry the column.

----Wash the sample with 1ml methanol, and the speed is 15drops/min.

----Evaporate the impregnant with 50~60C weak air or nitrogen gas flow.

----Dissolve the dry residue with 1ml deionized water.

----Take 20ml of the prepared solution for assay.

8.5 Feed sample

----Comminute the feed sample in the mortar, and weigh 2.0±0.05g comminuted sample into a 50ml polystyrene centrifuge tube, add 2ml 1M hydrochloric acid(see solution 5) and 16ml deionized water, then homogenize with homogenizer or stamocher.

----Whorl for 3min with gyroscope or vortex mixer, and shake for 15min with shaker.

----Centrifuge at least 3000g for 20min, transfer all the supernate into a 10ml clean polystyrene centrifuge tube, add 2ml 1M sodium hydrate (see solution 6) and mix completely, check whether the pH is between 6.5~7.5. (regulate the pH with sodium hydrate or hydrochloric acid)

---- Centrifuge at least 3000g, for 20min, transfer 100ml supernate into a 2ml clean polystyrene centrifuge tube, add 900ml deionized water and mix completely.

----Take 20ml for assayC

9. Assay process

9.1 Notice before assay:

9.1.1 Make sure all reagents and microwells are all at room temperature (20-25C).

9.1.2 Return all the rest reagents to 2~8C 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:

Notice before assay:

9.2.1 Take all reagents out at room temperature (20-25C) 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 2-8C immediately.

9.2.3 all reagents should be rewarmed 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 solution/sample: Add 20 µl of standard solution or prepared sample to corresponding wells. add 50ml enzyme-labeled secondary antibody solution, add 80µl antibody solution to each well, and then mix gently by shaking the plate manually and incubate for 30min at 25C with cover.

9.2.6 Wash: Remove the cover gently and pure the liquid out of the wells and rinse the microwells with 250µl diluted wash solution(see solution 10) at interval of 10s for 3~4 times. Absorb the residual water with absorbent paper.

9.2.7 Coloration: Add 50µl solution A and 50µl solution B to each well. Mix gently by shaking the plate manually and incubate for 20 min at 25C with cover(see 12.8).

9.2.8 Measure: Add 50µl the stop solution to each well. Mix gently by shaking the plate manually and measure the absorbance at 450nm against an air blank (It's suggested measure with the dual-wavelength of 450/630nm. Read the result within 5min after addition of stop solution.) (We can also measure by sight without stop solution in short of the ELIASA instrument)

10. Results

There are 2 different methods to determinate the results. Method 1 leads to a round estimation, and method 2 leads to definite quantity estimation. (Please notice: the absorption is inversely proportional to the Clenbuterol concentration in the sample.)

10.1 Round estimation

We can get the range of different strengths from the compare of average absorption and standards by sight. For example, the absorption of sample 1 is 0.301, sample 2 is 0.712, and the absorptions of Clenbuterol standard solutions: 1.952(0ppb) ; 1.420(0.1ppb); 0.980(0.3ppb); 0.464(0.9ppb); 0.256(2.7ppb); 0.152(8.1ppb). So we can say the strength of diluted sample 1 is between 0.9ppb and 2.7ppb; and diluted sample 2 between 0.3ppb to 0.9ppb. In order to obtain the Clenbuterol actually contained in a sample, the diluted sample results must be further multiplied by the corresponding dilution multiple.

10.2 Definite quantity estimation

(1) 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.

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

B0

B ——absorbance standard (or sample)

B0 ——absorbance zero standard

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

--- The Clenbuterol concentration of each sample (ppb), which can be read from the calibration curve, is multiplied by the corresponding dilution multiple of each sample followed, and the actual concentration of sample is obtained.

For evaluation of the ELISA kits special software has been developed for exact and rapid analysis. The analysis software can be ordered on request.

Dilution multiple of samples:

urine:1;

Tissue samples treated with Method (a):1;

Tissue samples treated with Method (b):4;

Feed samples:100;

11. Sensitivity, accuracy and precision

Test Sensitivity**:0.1ppb**

Detection limit**:**

Urine…… 0.1ppb

Tissue:

Tissues high in fat…… 0.1ppb

Method (a)…… 0.1ppb

Method (b)…… 0.4ppb

feed……10ppb

Accuracy:

urine…… 90%±15%

Tissue…… 70%±10%

feed……95%±15%

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 (20-25C).

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. Homogenize each reagent before use.

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, for it will drop the sensitivity.

12.6 Storage condition:

Keep the ELISA kits at 2-8C,do not freeze. Seal rest microwell plates Avoid straight sunlight during all incubations. Covering the microtiter plates is recommended for the standard sample and colorless chromogenic reagent are sensitive to light.

12.7 Indications for the reagents going bad:

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 20 min after adding Solution A and Solution B. And you can prolong the incubation time ranges from 20min to30 min if the color is too light to be determined., on the contrary, shorten the incubation time properly.

12.9 The optimal reaction temperature is 25C. Higher or lower temperature will lead to the changes of sensitivity and absorbance values.

13. Storage condition and storage period

Storage condition: 2-8C.

Storage period:6months.