Importance of β-agonist Determination

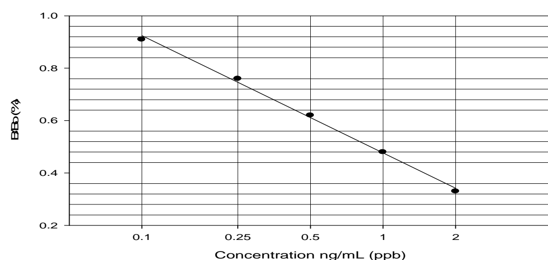
Veterinary residues in foods pose a serious threat to public health. β-agonists are a group of veterinary drugs that have been used illegally in some countries. β-agonists act by impeding the uptake of adrenal hormones by nerve cells and through the stimulation of the cardiac system. They alter body composition by redistributing fat from muscle tissue, resulting in higher production efficiencies. The monitoring of raw meat and animal feed for drug and chemical residues is necessary to ascertain that these compounds are not misused and do not present a danger to consumers.

The potential risk for human health posed by the presence of β-agonists is high, due to the severity of the possible adverse effects. The β-agonist clenbuterol has been implicated in many poisoning cases in European and Asian countries. Although urine is the most frequently analyzed sample matrix, other matrices, such as meat, milk and feed are also routinely analyzed.

The Abraxis β-agonist ELISA allows the determination of 41 samples in duplicate determination. Only a few milliliters of sample are required. The test can be performed in approximately 1 hour.

Performance Data

Test sensitivity: The limit of detection for Clenbuterol calculated as Xn +/- 3SD (n=20) or as 90% B/Bound is equal to 0.1 ng/mL. The concentration of residue necessary to cause 50% inhibition (50% B/B_0) is approximately 0.8 ng/mL. Determinations closer to the middle of the calibration range of the test yield the most accurate results.



Test reproducibility: Coefficients of variation (CVs) for standards: <10%; CVs for samples: <15%.

Selectivity: This ELISA recognizes several β-agonists with varying degrees:

Cross-reactivities:

Compound	Cross-reactivity
Clenbuterol	100%
Bromobuterol	95%
Cimaterol	95%
Salbutamol	80%
Ractopamine	80%
Tulobuterol	50%
Mabuterol	45%
Zilpaterol	20%
Phenethylamine A	115%

Samples: To eliminate matrix effects in urine or pork meat samples, sample clean-up and/or dilutions may be required. See Preparation of Samples section.

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β-agonist (BR) ELISA, Microtiter Plate

Enzyme-Linked Immunosorbent Assay for the Determination of β-agonists in Contaminated Samples

Product No. 515545

1. General Description

The β-agonist Broad Reactivity (BR) ELISA is an immunoassay for the detection of β-agonists in the following type of samples: muscle, liver, feed, serum (for urine use kit PN 515535). This test is suitable for the quantitative and/or qualitative detection of β-agonists in contaminated samples. Positive samples should be confirmed by HPLC, GC/MS, or other conventional methods.

2. Safety Instructions

The standard solutions in this test kit contain small amounts of Clenbuterol. In addition, the substrate solution contains tetramethylbenzidine and the stop solution contains diluted sulfuric acid. Avoid contact of stopping solution with skin and mucous membranes. If these reagents come in contact with the skin, wash with water.

3. Storage and Stability

The β-agonist ELISA should to be stored in the refrigerator (4–8°C). The solutions must be allowed to reach room temperature (20-25°C) before use. Reagents may be used until the expiration date on the box.

4. Test Principle

The test is a direct competitive ELISA based on the recognition of β-agonist compounds by specific antibodies. β-agonists, when present in a sample, and a β-agonist-enzyme conjugate compete for the binding sites of anti-β-agonist antibodies immobilized on the wells of a microtiter plate. After a washing step and addition of the substrate solution, a color signal is produced. The intensity of the blue color is inversely proportional to the concentration of β-agonist present in the sample. The color reaction is stopped after a specified time and the color is evaluated using an ELISA reader. The concentrations of the samples are determined by interpolation using the standard curve constructed with each run.

5. Limitations of the β-agonist ELISA, Possible Test Interference

Numerous organic and inorganic compounds commonly found in samples have been tested and found not to interfere with this test. However, due to the high variability of compounds that might be found in samples, test interferences caused by matrix effects can not be completely excluded. Mistakes in handling the test can also cause errors. Possible sources for such errors can be:

Inadequate storage conditions of the test kit, incorrect pipetting sequence or inaccurate volumes of the reagents, too long or too short incubation times during the immune and/or substrate reaction, extreme temperatures during the test performance (lower than 10°C or higher than 30°C).

The Abraxis β-agonist ELISA kit provides screening results. As with any analytical technique (GC, HPLC, etc.), positive samples requiring some action should be confirmed by an alternative method.

Working Instructions

A. Materials Provided

1. Microtiter plate coated with an antibody against *β*-agonist.
2. Clenbuterol Standards (6): 0, 0.1, 0.25, 0.5, 1.0, 2.0 ng/mL. .
3. β-agonist-HRP Conjugate (150X concentrate), 0.1 mL/vial.
4. Conjugate Diluent Solution (10X concentrate), 16 mL
5. Sample Diluent, 30 mL (ready to use). Use to dilute samples.
6. Wash Solution (5X) Concentrate, 50 mL.
7. Color (Substrate) Solution (TMB), 12 mL.
8. Stop Solution, 12 mL.

B. Test Preparation

Micro-pipetting equipment and pipette tips for pipetting the standards and the samples are necessary. We recommend using a multi-channel pipette or a stepping pipette for adding the conjugate, antibody, substrate and stop solutions in order to equalize the incubations periods of the solutions on the entire microtiter plate. Please use only the reagents and standards from one package lot in one test, as they have been adjusted in combination.

1. Adjust the microtiter plate and the reagents to room temperature before use.
2. Remove the number of microtiter plate strips required from the foil bag. The remaining strips are stored in the foil bag and zip-locked closed. Store the remaining kit in the refrigerator (4-8°C).
3. The standard solutions, antibody, substrate and stop solutions are ready to use and do not require any further dilutions.
4. Dilute the Conjugate Diluent Solution at a ratio of 1:10 with deionized or distilled water. If using 1 mL then dilute with 9 mLs.
5. The β-agonist -HRP Conjugate is provided as a 150X concentrate. Before each assay, calculate the volume of conjugate needed. Dilute only the amount necessary for the samples to be analyzed. Once diluted, the conjugate solution will only remain viable for one week (stored frozen). If additional samples are to be analyzed more than one week after reconstitution, a new vial of conjugate must be prepared. To dilute, add 40 µL of HRP Conjugate to a vial containing 6.0 mL of diluted Conjugate Diluent (1X) and vortex thoroughly before use, this dilution is good for 60 wells.
6. Dilute the wash buffer concentrate at a ratio of 1:5. If using the entire bottle (100 mL), add to 400 mL of deionized or distilled water.
7. The stop solution should be handled with care as it contains diluted H_2SO_4.
8. Tissue Extraction Solvent is prepared by adding 100 mL of DI water, 2.0 mL of 37% HCl into 900 mL of methanol.

C. Assay Procedure

1. Add 50 µL of the **standard solutions or samples** (sample extracts) into the wells of the test strips according to the working scheme given. We recommend using duplicates or triplicates.
2. Add 100 µL of diluted **enzyme conjugate** solution to the individual wells successively using a multi-channel pipette or a stepping pipette.
3. Incubate the strips for 30 minutes at room temperature.
4. After incubation, remove the covering and vigorously shake the contents of these wells into a sink. Wash the strips **four times** using the 1X washing buffer solution. Use at least a volume of 250 µL of washing buffer for each well and each washing step. Remaining buffer in the wells should be removed by patting the plate dry on a stack of paper towels.
5. Add 100 µL of **substrate (color) solution** to the wells. Cover the wells with parafilm or tape and mix the contents by moving the strip holder in a circular motion on the benchtop for about 30 seconds. Incubate the strips for 20 minutes at room temperature. Protect the strips from direct sunlight.
6. Add 100 µL of **stop solution** to the wells in the same sequence as for the substrate solution.
7. Read the absorbance at 450 nm using a microplate ELISA photometer within 15 minutes after the addition of the stopping solution.

D. Evaluation

The evaluation of the ELISA can be performed using commercial ELISA evaluation programs (4-Parameter (preferred) or Logit/Log. For manual evaluation, calculate the mean absorbance value for each of the standards. Calculate the $\%B/B_0$ for each standard by dividing the mean absorbance value for each standard by the Zero Standard (Standard 0) mean absorbance. Construct a standard curve by plotting the $\%B/B_0$ for each standard on the vertical linear (y) axis versus the corresponding β-agonist concentration on the horizontal logarithmic (x) axis on graph paper. $\%B/B_0$ for samples will then yield levels in ppb of a β-agonist by interpolation using the standard curve. Samples showing lower concentrations of β-agonist compared to Standard 1 (0.1 ng/mL) are considered as negative. Samples showing a higher concentration than Standard 5 (2.0 ng/mL) must be diluted further to obtain accurate results.

E. Additional Materials (not included with the test kit)

1. Micro-pipettes with disposable plastic tips (10-200 and 200-1000 µL)
2. Multi-channel pipette (10-250 µL) or stepper pipette with plastic tips (10-250 µL)
3. Microtiter plate washer (optional)
4. Microtiter plate reader (wave length 450 nm)
5. Shaker for microtiter plates (optional)
6. Centrifuge, capable of spinning at 3,500 X g
7. Timer
8. Tape or Parafilm
9. Glass vials or tubes, 4 and 10 mL capacity
10. **Tissue Extraction Solution**: 0.5 N and 1N Sodium Hydroxide, 3% Trichloroacetic acid (TCA), Dichloromethane, 1N HCl solution.
11. Nitrogen
12. Tube shaker or overhead rotator.

F. Working Scheme

The microtiter plate consists of 12 strips of 8 wells, which can be used individually for the test. The standards must be run with each test. Never use the values of standards which have been determined in a test performed previously.

Std 0-Std 5: Standards
0; 0.1; 0.25; 0.50; 1.0; 2.0 ppb

Sam1, Sam2, etc.: Samples

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std 0	Std 4	etc.									
B	Std 0	Std 4	etc.									
C	Std 1	Std 5										
D	Std 1	Std 5										
E	Std 2	Sam 1										
F	Std 2	Sam 1										
G	Std 3	Sam 2										
H	Std 3	Sam 2										

G. Preparation of Samples

1. Tissue Sample (Muscle, Liver)- Procedure 1. (Dilution Method)

1. Weigh 1.0 g of homogenized tissue (should have a paste-like consistency) into a 10mL or larger glass vial.
2. Add 3.0 mL of 3% TCA solution. Vortex for ½ minute, then place in a tube shaker or overhead rotator for 10 minutes.
3. Centrifuge vial for 15 minutes at 3500 5pm or 1,700 x g.
4. Pipette 0.5 mL of supernatant into a clean vial/tube, add 0.25 mL of 0.5 N NaOH and 0.25 mL of sample Diluent. Mix well.
5. Centrifuge vial/tube for 10 minutes at 3500 g
6. The upper clear solution is ready to be analyzed in the assay (Assay Procedure, step 1).

The β-agonist concentration contained in the tissue samples is then determined by multiplying the ELISA result by the dilution factor of 8. Highly contaminated samples outside of the calibration range of the assay must be diluted further and re-analyzed. **Sensitivity = 0.8 ppb**.

2. Procedure 2 (Extraction Method).

1. Weigh 1.0 g of homogenized tissue (should have a paste-like consistency) into a 10mL or larger glass vial.
2. Add 2.0 mL of Tissue Extraction Solvent. Vortex for ½ minute, then place in a tube shaker or overhead rotator for 10 minutes.
3. Centrifuge vial for 15 minutes at 3500 g.
4. Transfer 1.0 mL of supernatant into a clean 10 mL glass vial/tube.
5. Reduce to dryness under Nitrogen at 40-50 °C.
6. Re-dissolve using 1.0 mL of the Diluted Enzyme Conjugate diluent and 0.5 mL of dichloromethane, vortex well for 30 seconds. **NOTE:** If the sample is liver dissolve with 2.0 mL of diluted Enzyme Conjugate diluent.
7. Centrifuge vial for 10 minutes at 3500 rpm or 1,700 x g
8. Transfer 0.5 mL of upper phase to another glass tube, and add 0.25 mL of n-Hexane. Vortex throughlly.
9. Centrifuge vial/tube for 10 minutes at 3500 rpm or 1,700 x g
10. The bottom layer is ready to be analyzed in the assay (Assay Procedure, step 1).

The β-agonist concentration contained in the tissue samples is then determined by multiplying the ELISA result by the dilution factor of 2 (4 for liver). Highly contaminated samples outside of the calibration range of the assay must be diluted further and re-analyzed. **Sensitivity = 0.2 ppb (Liver 0.4 ppb)**.

3. Feed

1. Grind feed sample to a fine powder.
2. Weigh 1.0 g of the powdered feed and add 0.5 mL of 1N HCl and 4.5 mL of deionized water.
3. Vortex for ½ minute, then place in a tube shaker or overhead rotator for 15 minutes.
4. Centrifuge vial/tube for 15 minutes at 3500 rpm or 1,700 x g.
5. Transfer supernatant into a clean glass vial/tube and add 0.25 mL of 1N NaOH.
6. Vortex for ½ minute. Then centrifuge vial for 15 minutes at 3500 rpm or 1,700 x g.
7. Transfer 0.10 mL of the upper clear solution and add 0.30 mL of the diluted Conjugate Diluent (1X).
8. Analyze in the assay (Assay Procedure, step 1).

The β-agonist concentration contained in the feed samples is then determined by multiplying the ELISA result by the dilution factor of 20. Highly contaminated samples outside of the calibration range of the assay must be diluted further and re-analyzed. **Sensitivity = 2.0 ppb.**

4. Serum

1. Transfer 0.50 mL of serum sample to a tube containing 1.0 mL of diluted Enzyme Conjugate Diluent (1X) and vortex to mix.
2. Analyze in the assay (Assay Procedure, step 1).

The β-agonist concentration contained in the serum samples is then determined by multiplying the ELISA result by the dilution factor of 3. Highly contaminated samples outside of the calibration range of the assay must be diluted further and re-analyzed. **Sensitivity = 0.30 ppb.**