Importance of β-agonist Determination

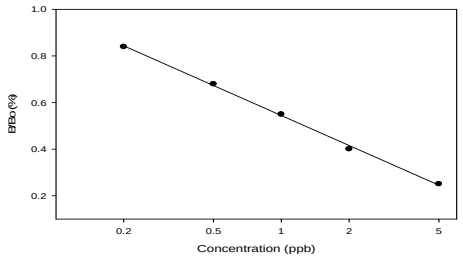
Veterinary residues in foods pose a serious threat to public health. β-agonists are a group of veterinary drugs that have been used illegally in some countries. β-agonists act by impeding the uptake of adrenal hormones by nerve cells and through the stimulation of the cardiac system. They alter body 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 less than 2 hours.

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 1.25 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:

Brombuterol	119%
Clenbuterol	100%
Salbutamol	39%
Terbutaline	38%
Mapenterol	15%
Cimaterol	4%

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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For ordering or technical assistance contact:

Abraxis LLC
54 Steamwhistle Drive
Warminster, PA 18974
Tel.: (215) 357-3911
Fax: (215) 357-5232
Email: info@abraxiskits.com
WEB: www.abraxiskits.com

R033109

β-agonist ELISA, Microtiter Plate

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

Product No. 5061A

1. General Description

The β-agonist ELISA is an immunoassay for the detection of β-agonists. 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 rabbit anti-β-agonist antibodies in solution. The β-agonist antibodies are then bound by a second antibody (goat anti-rabbit) immobilized on the 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 a second antibody (goat anti-rabbit).
2. Clenbuterol Standards (6): 0, 0.2, 0.5, 1.0, 2.0, 5.0 ng/mL.
3. Antibody Solution (rabbit anti-β-agonist), 6 mL.
4. β-agonist-HRP Conjugate, 6 mL.
5. Sample Diluent, 25 mL. Use to dilute samples.
6. Wash Solution (5X) Concentrate, 100 mL.
7. Color (Substrate) Solution (TMB), 16 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, enzyme conjugate, antibody, substrate and stop solutions are ready to use and do not require any further dilutions.
4. 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.
5. The stop solution should be handled with care as it contains diluted H_2SO_4.

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 50 µL of **enzyme conjugate** solution to the individual wells successively using a multi-channel pipette or a stepping pipette.
3. Add 50 µL of **antibody solution** to the individual wells successively using a multi-channel pipette or a stepping pipette. 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. Be careful not to spill contents.
4. Incubate the strips for 60 minutes at room temperature.
5. After incubation, remove the covering and vigorously shake the contents of these wells into a sink. Wash the strips **three 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.
6. Add 150 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 30 minutes at room temperature. Protect the strips from direct sunlight.
7. Add 100 µL of **stop solution** to the wells in the same sequence as for the substrate solution.
8. 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 (0.2 ng/mL) are considered as negative. Samples showing a higher concentration than Standard 5 (5.0 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. Timer
7. Tape or Parafilm

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; 0.2; 0.5; 1.0; 2.0; 5.0 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

Pork Extraction

1. Weigh 1g of homogenized pork (should have a paste-like consistency) into a 10mL or larger glass vial.
2. Add 4mL of 0.2M Tris/HCL/Pronase buffer solution. Mix gently by inversion, **do not vortex**.
3. Incubate mixture overnight with constant stirring in a 55ºC water bath.
4. Centrifuge vial for 15 minutes at 3500 g. Pipette 2mL of supernatant into a clean vial.
5. Adjust pH to 9.40 - 9.60 with 6N NaOH.
6. Add 4mL of Isobutanol and vortex for 30 seconds. Allow phase separation (approximately 5 minutes).
7. Pipette 2mL of top layer into a clean vial and evaporate to dryness at 40- 60ºC under a gentle stream of nitrogen.
8. Add 25µl of MeOH and vortex thoroughly to re-dissolve, then add 225µl of 20mM PBS. Vortex.
9. Dilute 1:32 (i.e. 50 uL of extract and 1.55 mL of diluent) with sample diluent (10% MeOH in 20 mM PBS, pH 7.4), then analyze as samples (Assay Procedure, step 1).

The β-agonist concentration contained in the pork samples is then determined by multiplying the ELISA result by the dilution factor of 32. Highly contaminated samples outside of the calibration range of the assay must be diluted further and re-analyzed.

Urine Sample Preparation

Urine samples must be diluted 1:10 in sample diluent (10% MeOH in 20 mM PBS, pH 7.4) prior to analysis to eliminate interferences. The β-agonist concentration contained in urine samples is then determined by multiplying the ELISA result by the dilution factor of 10. Samples with low concentrations of β-agonists or samples which must meet specific regulatory levels may be analyzed without dilution after the following sample clean-up:

1. Add 1mL of urine to a clean glass vial. Adjust pH to 8 with NaOH.
2. Add 4mL of methyl t-butyl ether (MTBE). Vortex.
3. Centrifuge vial for 2 minutes at 10,000 g. Allow phase separation (approximately 5 minutes).
4. Pipette 2mL of top layer into a clean vial.
5. Evaporate to dryness at 40-50ºC under a gentle stream of nitrogen.
6. Add 250µl of sample diluent and vortex thoroughly to re-dissolve, then analyze as samples (Assay Procedure, step 1).

The β-agonist concentration contained in urine samples when using the sample clean up procedure is then determined by dividing the ELISA result by the concentration factor of 2. Highly contaminated samples outside of the calibration range of the assay must be diluted further and re-analyzed.

For additional extraction procedures for various matrices please contact Abraxis LLC.

Preparation of Solutions:

0.2M Tris/HCL buffer, pH 8.0: Dissolve 24.4 g of Tris and 14.7 g of Calcium Chloride in 1 L of deionized or distilled water. Adjust pH to 8.0 with 6N HCL.

0.2M Tris/HCL/Pronase buffer: Dissolve 5 mg of Pronase in 16 mL of 0.2M Tris/HCL buffer. **Mix gently**.

20mM Phosphate Buffered Saline (PBS), pH 7.4: In 800 mL of distilled or deionized water, dissolve 2.277 g of Sodium Phosphate Dibasic anhydrous, 0.548 g of Sodium Phosphate Monobasic monohydrate, and 18.0 g of Sodium Chloride. Bring to 1 L volume with distilled or deionized water. pH= 7.2-7.4.