β-Agonists ELISA kit (Clenbuterol)

Enzyme immunoassay for the detection of β-agonists (Cat.nr. HU0050018)

β-agonists (clenbuterol) ELISA kit is a kit prepared for an immunoenzymatic assay for the quantitative analysis of β-agonists. The kit contains the procedure and the materials sufficient for 96 determinations, including standards.
For result evaluation a microtiter plate or a strip photometer is required (manual or automatic ELISA reader).

Type of samples that can be analyzed (matrices)
Human, bovine, equine urines and serum, eyes, liver, feed, milk and milk powder.

Sample preparation
- Urine, serum: centrifugation or filtration
- Feed: grinding, acid extraction, centrifugation
- Milk, milk powder: dilution, acid extraction, centrifugation, dilution
- Liver: homogenization, acid extraction, centrifugation, neutralization.
- Eye: enzymatic digestion, centrifugation.

Assay time: 45 minutes (sample preparation not included).

Detection limit
- Urine, serum: 0.15 ppb
- Liver: 0.3 ppb
- Eye: 0.75 ppb
- Feed, milk, milk powder: 7.5 ppb.

Specificity	
Compound	**Cross-reactivity**
Clenbuterol	100%
Mabuterol	67%
Salbutamol	20%
Terbutaline	15%
Cimaterol	5%
Methylclenbuterol	9%
Hydroxymethylclenbuterol	6%

1. TEST PRINCIPLE

The assay is performed in plastic microwells which have been coated with goat anti-rabbit IgG. Clenbuterol standard or the sample solution, the enzyme conjugate and rabbit anti-β-agonists antibodies are added to the microwells. During the first incubation, free and enzyme-labelled β-agonists compete for the anti-β-agonists antibodies binding sites; moreover, anti-clebuterol antibodies are bound to the solid fase by the immobilised anti-rabbit IgG. The bound enzyme activity is determined adding a fixed amount of a chromogenic substrate. The enzyme converts the colourless chromogen into a blue product.
The addition of the stop reagent leads to a colour change from blue to yellow. The absorbance is measured with a microplate reader at 450 nm.
The colour development is inversely proportional to the β-agonists concentration in the standard solution /sample.

2. PROVIDED REAGENTS

Microtiter plate:96 wells (12 strips X 8 wells) coated with goat anti-rabbit IgG.
As the strips are breakable, the wells can be used individually. For this purpose, it is sufficient to take out the wells from the frame and to break the joint.
Clenbuterol standard: 7 plastic vials containing 1.5 ml: 0 ng/ml; 0.15 ng/ml, 0.3 ng/ml, 0.6 ng/ml, 1.25 ng/ml, 2.5 ng/ml and 5 ng/ml.
Anti β–agonist antibody:1 plastic bottle containing 12 ml. Blue solution.
Enzyme conjugate:1 bottle vial containing 12 ml. Red solution.
Washing-buffer 10X:1 plastic bottle containing 50 ml.
Developing solution:1 plastic bottle containing 24 ml.
Stop solution:1 glass bottle containing 8 ml. White cap.

3. REQUIRED BUT NOT PROVIDED MATERIALS

- Distilled water
- HCl (feed, milk, milk powder, liver)
- NaOH (feed, milk, milk powder, liver)
- Protease (Subtilisin Carlsberg protease, es. cod. Sigma P5380; for eye).
- 0.1 M phosphate buffer pH 8 (for eye).

Equipment
- Homogenizer (liver)
- Bench-centrifuge (relative centrifugal force requested: 2000 xg for feed, optional for urine and serum; 6000 xg for milk and milk powder, 10000 xg for liver and eye)
- Grinder (feed)
- Low speed shaker (400 rpm; for feed)
- Balance.
- Filterpaper Whatman 01 (for urine and serum, in alternative to centrifuge)
- pHmeter or pH indicator strips
- Incubator (40°C for milk and milk powder; 56°C and 100°C for eye).
- Scalpel
- Vortex
- 20-200 µl, micropipettes, tips.
- 100-1000 µl, micropipettes, tips.
- 50-250 µl, multichannel micropipette, tips.
- Microplate reader, filter 450 nm

4. PRECAUTIONS

- The test is for *in vitro* diagnostic use only.
- Some reagents contain solutions that may be identified as dangerous substances by the Regulation (EC) *N° 1272/2008.*

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Please refer to Material Safety Data Sheet available on both the Eurofins Technologies and Eurofins Tecna (tecna.eurofins-technologies.com) web site.

- Handle the reagents with caution, avoiding contact with skin, eyes and mucous membranes.

5. HANDLING AND STORAGE INSTRUCTIONS

- Store the kit at +2/+8 °C and never freeze.
- **Bring all reagents to room temperature before use (at least 1 hour). ATTENTION:** Do not unseal the microplate until it reaches the room temperature.
- Reseal the unused strips of the microtiter plate in the bag together with the desiccant bag provided.
- Return all reagents to +2/+8 °C immediately after use.
- Do not use components after the expiration date.
- Do not intermix components from different kit lots.
- Do not use photocopies of the instruction booklet. Follow the original instruction booklet that is included with the kit.
- Do not change the assay procedure, in particular:
  - do not prolong the incubation times,
  - do not incubate the plate at temperatures higher than 25°C,
  - do not shake the plate during the incubations.
- Use accurate and precise micropipettes with suitable tips for dispensing.
- Once started, complete all the steps without interruption.
- The reproducibility of ELISA results depends largely upon the efficiency and uniformity of microwells washing; always keep to the described procedure.
- Use a single disposable tip for each standard solution and sample to avoid cross-contamination.
- Do not allow tips to contact the liquid already in the microwells.
- Avoid exposure to direct light during all incubations. It is recommended to cover the microtiter plate without using plate sealers.

6. SAMPLES PREPARATION

6.1 Urine and serum samples

1) Centrifuge the samples at 2000 g for 5 minutes or filtrate them;
2) Use the clean supernatant in the test.
3) The dilution factor is 1.

6.2 Feed

1) Finely grind the feed;
2) Weigh 1 g of powder in a 50 ml tube;
3) Add 50 ml of HCl 0.01 M;
4) Mix thoroughly for at 350-400 rpm for 5 minutes;
5) Check the pH to enter in the 6.5 - 8 range, otherwise adjust the pH with HCl or NaOH 1M;
6) Centrifuge at 2000 g for 5 minutes or filtrate;
7) Use the clean supernatant directly in the test.
8) The dilution factor is 50.

6.3 Milk and powder milk

1) Weigh 1 g of milk powder (or 1 ml of milk) in a 50 ml tube;
2) Add 3 ml (2 ml for milk) of distilled water in a 50 ml tube;
3) Add 100 ul of HCl 5 M (up to pH 3)
4) Warm up to 40 °C for 3-5 minutes until milk curles;
5) Centrifuge at 2000 g for 5 minutes;
6) Add 100 µl (two drops) NaOH 5M;
7) Add 46.8 ml of distilled water;
8) Centrifuge at 6000 g for 15 minutes;
9) Use the clean supernatant directly in the test.
10) The dilution factor is 50.

6.4 Liver

1) Homogenise with Ultraturrax or similar equipment;
2) Weigh 1 g of homogenized liver in a 10 ml test tube;
3) Add 1 ml of HCl 0.01 M;
4) Vortex for 2 minutes;
5) Centrifuge at 10000 g for 5 minutes or at 3000 g for 15 minutes;
6) Transfer 250 µl of the clean supernatant to a 1,5 ml tube;
7) Adjust the pH around 7 adding 5 µl of NaOH 2N;
8) Centrifuge at 10000 g for 5 minutes or at 3000 g for 15 minutes;
9) Use the clean supernatant for the test;
10) The dilution factor is 2.

ATTENTION: Perform the assay immediately after the last centrifugation.

6.5 Eye

1) Remove from the eye, the aqueous humor, vitreous humour cornea, lens and retina;
2) Scrape the choroid from the everted eye ball with a scalpel, so that choroid is associated with pigmental retinal epithelium (PRE) layer;
3) Weigh 0.1 g of choroid/PRE in a polythene microtube;
4) Add 0.4 ml of phosphate buffer 0.1M, pH 8.0;
5) Add 10 µl of Subtilisin Carlsberg protease from a stock solution of 20mg/ml (stored at -20°C);
6) Incubate at 56°C for 1h;
7) Incubate at 98-100° C for 15 minutes;
8) Centrifuge the resulting inky suspension at 10000 x g for 5 minutes (or 3000 x g for 15 minutes);
9) The clear amber supernatant is ready.
10) The dilution factor is 5.

7. WORKING SOLUTIONS PREPARATION

Clenbuterol standard solutions: ready to use;
Enzyme conjugate: ready to use;
Anti β–agonist-antibody: ready to use;
Washing buffer: dilute the concentrated buffer 1:20 (1+19) with distilled water. **ATTENTION**: In presence of crystals, bring the solution at room temperature and stir in order to solve them completely.
The diluted washing buffer is stable at room temperature for 24 hours and at +2/+8°C for two weeks.
Developing solution: ready to use; this solution is light sensitive: keep away from direct light.
Stop solution: ready to use. **ATTENTION**: It contains 1M sulphuric acid. Handle with care; in case of contact flush immediately with plenty of water.

8. ASSAY PROCEDURE

1) Predispose an assay layout, recording the standard and samples positions, taking into account that all have to be run in duplicate.
2) First incubation
   - Add 20 µl of each standard/ sample into the corresponding wells.

- Using the multichannel pipet, add 80 µl of enzyme conjugate in each well.
- Using the multichannel pipet, add 100 µl of anti-β-agonists antibody in each well.
- Shake the plate gently with rotatory motion for few seconds.
- Incubate 30 minutes at room temperature.
- Do not prolong the first incubation time and do not use automatic shakers.

3) Washing
   - Pour the liquid out from the wells.
   - Fill completely all the wells with working wash solution using a squeeze bottle. Pour the liquid out from the wells.
   - Repeat the washing sequence five (5) times.
   - Remove the remaining droplets by tapping the microplate upside down vigorously against absorbent paper.

Do not allow the wells to dry out

4) Developing
   - Using the multichannel pipet, add 200 µl of developing solution to each well.
   - Mix thoroughly with rotatory motion for few seconds.
   - Incubate for 15 minutes at room temperature.
5) Using a multichannel pipet, add 50 µl of stop solution to each well and mix thoroughly with rotatory motion for few seconds.
6) Measure the absorbance at 450 nm.
7) Read within 60 minutes.

9. RESULTS CALCULATION

- Calculate the mean absorbance of each standard and sample.
- Divide the mean absorbance value of each standard and sample by the mean absorbance of the standard 0 (B_0) and multiply by 100; the standard 0 is thus made equal to 100% and all the other absorbance values are expressed as percentages:

$$\frac{\text{Standard (or sample) absorbance}}{\text{Standard 0 } (B_0) \text{ absorbance}} \times 100 = \frac{B}{B_0}\ (\%)$$

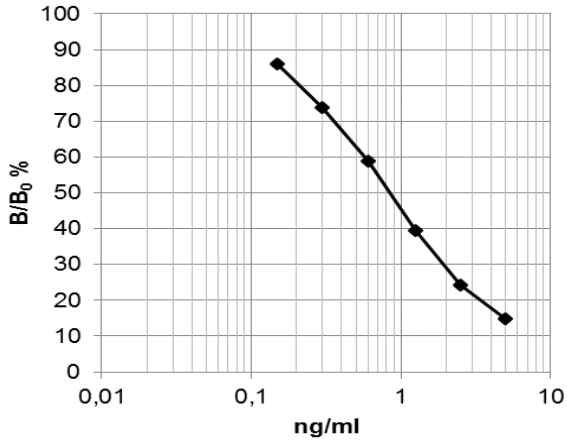
- Enter the B/B_0 values calculated for each standard in a semi-logarithmic system of coordinates and draw the standard curve.
- Interpolate the B/B_0 value of each sample to the corresponding concentration from the calibration curve. Multiply this concentration for the dilution factor, as reported in chapter 6.

Please note: To elaborate the ELISA results using the "point to point" method, Excel spreadsheet are available on the Eurofins Tecna website and can be downloaded directly from the bottom of the product page.

10. ASSAY LIMITATIONS

It is impossible to assay rabbit serum or tissues because the IgG of the sample would bind to the wells, replacing anti-β-agonists antibodies.
These matrices have to be analysed by another analytical method.

11. EXAMPLE OF CALIBRATION CURVE



12. RESULTS EVALUATION

After processing the results, it is necessary to verify the assay performance. The verification is performed by comparison of obtained data with those given in kit specifications (see chapter 13).
If the values are outside the specifications given, then the results of the test are not assured, therefore the β-agonists concentration levels in the samples may not be valid.
In these cases it is advised to check the expiry date of the kit, the wavelength at which the reading was performed, as well as the procedure followed.
If operation errors are not identified as cause, contact our technical assistance.
In order to avoid false positive results it is necessary to adopt a decision limit (CCα). The decision limit varies according to the type of sample. It is suggested to determine a decision limit for each matrix routinely analysed in your laboratory.

WARNING: Kit replacement will only be possible in case of return. The kit must be stored in its integral version at +2/+8°C.

13. KIT SPECIFICATIONS

13.1 Assay specifications

Description	Specifications
Mean B_0 absorbance	≥ 0.7 OD_{450nm}
B/B_0 50 %	0.8 – 2.1 ng/ml
Std duplicates mean C.V.	≤ 6 %

13.2 Assay performances

The kit performances hereby presented are from an in-house validation; Detection Capability (CCβ) was calculated as requested by of EU Decision 657/2002.

CCβ Detection capability	
Matrix	**Clenbuterol (ppb)**
Bovine liver	0.5
Bovine Urine	0.5

14. LIABILITY

Samples evaluated as positive using the kit have to be re-tested with a confirmation method.
Eurofins Technologies Hungary shall not be liable for any damages to the customer caused by the improper use of the kit and for any action undertaken as a consequence of results.
Eurofins Technologies Hungary shall not be liable for the unsafe use of the kit out of the current European safety regulations.

15. LITERATURE

L. Cerni, G. Biancotto, A. Tondolo and P. Bogoni: " Dexametasone and clenbuterol detection by enzyme immunoassay in bovine liver tissue: A new mutiresidue extraction procedure", Food and Agricultural Immunology (1998), vol 10, 307-315.

Angeletti, M. Paleologo Oriundi, R. Piro and R. Bagnati: "Application of an enzyme linked immunosorbent assay kit for b-agonists screening of bovine urines in north-eastern Italy", Analytica Chimica Acta (1993), vol. 275, 1-2, 269-274.

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