Enzyme immunoassay for the detection of β-agonists

(code AA107)

I'screen β-agonists v2 (code AA107) is a kit prepared for an immunoenzymatic assay for the quantitative analysis of β-agonists. The kit contains the procedures and materials sufficient for 96 determinations (including standards). A microtiter plate photometer or a strip photometer is required.

Analysable samples
Urine, liver, muscle, feed, milk powder, eye, and hair.

Sample preparation
Urine (method I): centrifugation, dilution
Liver (method I): homogenization, acid extraction, centrifugation, neutralization, centrifugation, dilution
Muscle: homogenization, extraction, centrifugation, evaporation, resuspension
Eye: protein digestion, centrifugation
Feed: acid extraction, centrifugation
Milk powder: resuspension, acid extraction, neutralization, centrifugation
Hair: washings, hydrolysis, extraction, centrifugation, evaporation, resuspension.
Urine and liver (method II): homogenization (for liver), enzyme acid digestion, extraction with organic solvent.

Assay time: 1 hour and 20 minutes (sample preparation not included).

Detection limit
Urine (method I), eye, hair: 0,185 ppb
Liver (method I): 0,296 ppb
Muscle: 0,092 ppb
Feed and milk powder: 3,7 ppb
Urine (method II): 0,037 ppb
Liver (method II): 0,074 ppb

Specificity*	
Compound	**cross-reactivity %**
Clenbuterol	100
Cimbuterol	96
Salbutamol	80
Bromobuterol	77
Mabuterol	55
Carbuterol	55
Clenpenterol	55
Mapenterol	45
Clenproperol	35
Terbutaline	30
Cimaterol	11
Fenoterol	< 0.07
Ractopamine	< 0.07
Isoxsuprine	< 0.07

* The crossreactivities are calculated at B/Bo of 80%

1 TEST PRINCIPLE
The assay is performed in plastic microwells which have been coated with rabbit anti-β-agonists antibodies (IgG). Clenbuterol standards or the sample solution and the enzyme conjugate are added to the microwells. During the incubation, free and enzyme-labelled β -agonists compete for the anti- β -agonists antibodies binding sites. Any unbound enzyme conjugate is then removed in a washing step. The bound enzyme activity is determined by 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 color change from blue to yellow. The absorbance is measured by a microplate reader at 450 nm. The colour development is inversely proportional to the β agonists concentration in the sample.

2 PROVIDED REAGENTS
Microtiter plate:96 wells (12 strips X 8 wells) coated with rabbit anti-β-agonists antibodies.
As the strips are breakable, the wells can be used individually.

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For this purpose, it is sufficient to take out the wells from the frame and to break the joint.
Clenbuterol standards: 6 plastic vials containing 1,5 ml: 0 ng/ml; 0,037 ng/ml; 0.075 ng/ml, 0.15 ng/ml, 0.6 ng/ml, 2.5 ng/ml.
Enzyme conjugate: 1 amber plastic vial containing 8 ml.
Dilution buffer 1 X: 1 plastic bottle containing 50 ml.
Washing buffer 10 X: 1 plastic bottle containing 50 ml.
Developing solution: 1 amber plastic bottle containing 14 ml.
Stop solution: 1 glass vial containing 8 ml. White cap.

3 REQUIRED BUT NOT PROVIDED MATERIALS

- Distilled water

MUSCLE: isobutanol, 1M Sodium Carbonate buffer (pH 9.8), 1M NaOH
HAIR: ethyl acetate, 1M NaOH, 1% (w/v) sodium dodecyl-sulfate 1%
EYE: 0.1M Phosphate buffer (pH 8.0), Protease (Sigma, code P5380)
LIVER (method I): 2M NaOH, 0,01M HCl, 0,1M HCl
LIVER (method II): 0,1M HCl, 0,2M acetate buffer pH 4,8, 2M NaOH, *Helix pomatia* juice (Sigma, code G0876), 1M carbonate buffer (pH 9,8), isobutanol
MILK: 5M NaOH, 5M HCl
FEED: 0,01M HCl
URINE (method II II): 0,2M acetate buffer pH 4,8, 1M HCl, *Helix pomatia* juice (Sigma, code G0876), 0,25 M carbonate buffer (pH 9.8), 1M NaOH, isobutanol.
Equipment

- Bench-centrifuge (all matrices)
- Balance (solid matrices)
- Grinder (feed)
- Ultra-turrax (muscle and liver)
- Vortex
- Shaker (hair)
- Scalpel (eye)
- Evaporator (muscle, milk powder, hair, method II for urine and liver)
- Incubator (eye, milk powder , hair, method II for urine and liver
- pH indicator strips
- 20-200 µl micropipette with tips.
- 50-200 µl multichannel micropipette with tips.
- Microtiter plate or strip reader equipped with a 450 nm filter.

4 PRECAUTIONS

- For *in vitro* diagnostic use only.
- Some reagents contain solutions that may be identified as dangerous substance by the Regulation (EC) No 1272/2008. Please refer to Safety Data Sheet available on Tecna 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;
- Close tightly using an adhesive-tape the unused strips of the microtiter plate in the bag together with the desiccant bag provided.
- Do not use components after the expiration date.
- Do not intermix components between different kit lots.
- Do not use photocopies of the instruction booklet. Keep always to the instruction booklet which is included inside the kit.
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6 SAMPLES PREPARATION

6.1.1 Urine (method I)

Centrifuge the samples at 2000g for 5' or filtrate them. Dilute the clean supernatant 5 times (1+4) with dilution buffer.

6.1.2 Urine (method II)

To 2 ml of urine, add 2 ml of 0,2M acetate buffer (pH 4,8). If necessary, to adjust pH to 4,8, use 1M HCl. Add 20 ul of *Helix pomatia* juice. Incubate ON at 37°C (or 2 hours at 55°C). After incubation, wait until RT is reached, then add 2 ml of 0,25M carbonate buffer (pH 9,8). Adjust the pH at 9,8 ± 0,2 by using 1M Na OH. Take 1,5 ml of the digested urine and add 2 ml of isobutanol. Mix with vortex for 1 minute. Centrifuge at 2000g for 5 minutes. Take 1 ml of the upper organic phase, and evaporate it to dryness at 50°C, under air stream. Mixing with vortex, reconstitute with 250 ul of dilution buffer.

6.2.1. Liver (method I)

Homogenise with Ultraturrax or similar equipment. Weigh 1 g of homogenized liver in a 10 ml test tube. Add 1 ml of HCl 0.01 M and vortex for 2'. Centrifuge at 10000 g. Transfer 250 ul of the clean supernatant to a 1,5 ml tube. Adjust the pH around 7 adding 5 ul of NaOH 2M. Centrifuge at 10000g for 5'. Dilute the clean supernatant 4x (1+3) with the dilution buffer.

6.2.1. Liver (method II)

Homogenise with Ultraturrax or similar equipment. Weigh 1 g of homogenized liver and add 5 ml of 0,1M HCl; mix with vortex for 1 minute. Centrifuge at 10000 for 5 minutes. Take 2 ml of supernatant and add 2 ml of 0,2M acetate buffer (pH 4,8). If necessary, to adjust pH to 4,8, use 2M NaOH. Add 20 ul of *Helix pomatia* juice and incubate ON at 37°C After incubation, wait until RT is reached, then to adjust pH, add 0,25 ml of 1M carbonate buffer (pH 9,8). If necessary, adjust the pH at 9,8 ± 0,2 by using 2M NaOH. Add 4 ml of isobutanol. Mix with vortex for 30 seconds. Centrifuge at 5000g for 5 minutes. Take 2 ml of the upper organic phase, and evaporate it to dryness at 50°C, under air stream. Mixing with vortex, reconstitute with 400 µl of dilution buffer. Centrifuge 3 minutes at 2000g and use the supernatant in the assay.

6.3 Eye

Remove from the eye, the aqueous humor, vitreous humour cornea, lens and retina; scrape the choroid from the everted eye ball with a scalpel, so that choroid is associated with pigmental retinal epithelium (PRE) layer. Weigh 0.1 g of choroid/PRE in a polythene microtube; add 0.4 ml of phosphate buffer 0.1M, pH 8.0 and 10µl of protease (Sigma, code P5380) from a stock solution of 20mg/ml (stored at -20°C); incubate at 56°C for 1h and then at 98-100° C for 15 min. Centrifuge the resulting inky suspension at 10000 x g for 5 min (or 3000 x g for 15 min). The clear amber supernatant is ready.

6.4 Feed

Finely grind the feed; weigh 1 g of powder and add 100 ml of 0.01 M HCl; mix thoroughly for 5'; Check the pH to enter in the 6.5 - 8 range, otherwise adjust the pH with

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diluted solutions of HCl or NaOH; centrifuge an aliquot of 1 ml at 2000 g for 5' or filtrate; use the clean supernatant directly in the test.

6.5 Muscle

Add 4 ml of 0.01M HCl to 1g of sample and homogenise with Ultra-Turrax or similar equipment; centrifuge 10 minutes at 4°C ,4000g. Transfer 2ml of the supernatant in another tube and add 0.25ml of 1M carbonate buffer (pH 9.8), adjust pH to 9.8 with 1M NaOH, if necessary. Add 4ml of isobutanol, mix (by vortex) for 30 seconds and centrifuge for 5 minutes at 2000g. Pipette 2ml of the upper organic layer into a glass tube and evaporate until dryness under a stream of air or nitrogen at 50°C. Resuspend the pellet in 500 µl of water.

6.6 Milk powder

Weigh 0,5 g of milk powder in a 50 ml tube. Add 3 ml of distilled water and mix until dissolved. Add 100 l of 5 M HCl (if possible, measure the pH and adjust to 3). Incubate at 40°C for 3 to 5 minutes until curdles. Add 100 l (2 drops) of 5M NaOH and 46,8 ml of distilled water. Centrifuge at 2000g for 15 minutes. Take 1 ml of supernatant and centrifuge it at 10.000g for 10 minutes. Use the supernatant directly in the test.

6.7 Hair

Weigh 0,5 g of hair and wash twice with 5 ml of 1% sodium dodecyl sulfate (w/v) and twice with 10ml of distilled water. Hydrolyze with 3ml of 1N NaOH for 30 minutes at 60°C. Cool at room temperature, add 7 ml of ethyl acetate and shake for 20 minutes (250-300 rpm). Centrifuge for 10 minutes at 2000g. Transfer 2.8 ml of the organic phase and evaporate until dryness under a stream of air or nitrogen at 50°C. Resuspend the pellet in 1 ml of dilution buffer.

7 WORKING SOLUTIONS PREPARATION

Clenbuterol standard: ready to use;
Enzyme conjugate: ready to use;
Dilution buffer 1 X: ready to use;
Washing buffer: dilute the concentrate 1:10 (1+9) with distilled water. ATTENTION: if crystals are present, 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. Caution: it contains 1 M sulfuric acid. Handle with care and in case of contact wash thoroughly with tap water.

8 ASSAY PROCEDURE

8.1 Preliminary comments

- Bring all reagents to room temperature before use.
- Return all reagents to +2/+8 °C immediately after use.
- Do not change the assay procedure, in particular:
  - do not prolong the first incubation time
  - do not incubate the plate at a temperature > 25°C or < 18°C;
  - do not shake the plate during the incubations;
  - use for dispensing accurate and precise micropipettes with suitable tips.
- 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 and sample to avoid cross-contamination.
- Do not allow tips to contact the liquid already in the microwells or the internal microwells surface.
- Avoid direct sunlight during all incubations. It is recommended to cover the microtiter plate without using sealing tapes.

8.2 Assay procedure

1. Predispose an assay layout, recording standard and samples positions, taking into account that all have to be run in duplicate.
Remove the strips not to be used from the frame; replace them in the pouch with the desiccant gel; reseal the pouch by the clump provided.
2. Add 50 µl of each standard/sample into the standard/sample wells
3. Using the multichannel micropipette, add 50 µl of enzyme conjugate in each well and shake the plate gently with rotatory motion for few seconds.
4. Incubate 60 minutes at room temperature;
Do not prolong the first incubation time and do not use automatic shakers
5. Washing sequence
- After incubation, 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 four (4) times.
Remove the remaining droplets by tapping the microplate upside down vigorously against absorbent paper;

Do not allow the wells to dry out.
6. Developing
- Using the multichannel micropipette, add 100 µl of developing solution to each well and mix thoroughly with rotatory motion for few seconds;

7. Incubate for 20 minutes at room temperature;
8. Using the multichannel micropipette, add 50 µl of stop solution to each well and mix thoroughly with rotatory motion for few seconds;
9. Measure the absorbance at 450 nm.
Read within 60 minutes.

9 RESULTS CALCULATION

- Calculate the mean absorbance of the each standards and samples;
- Divide the mean absorbance value of each standard and sample by the mean absorbance of the Standard 0 (B_O) and multiply by 100; the Maximum Binding is thus made equal to 100% and the absorbance values are quoted in percentage:

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

- Enter the B/B_O values calculated for each standard in a semi-logarithmic system of coordinates against the standard clenbuterol concentration; draw the standard curve.

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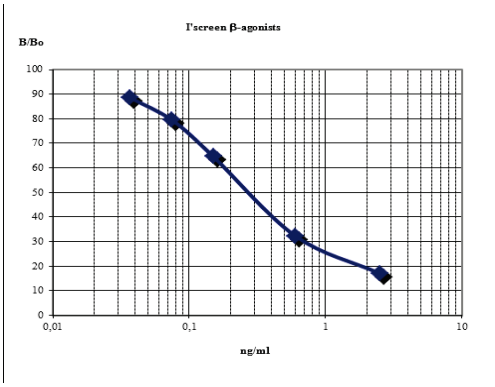
- Take the B/B_O value for each sample and interpolate the corresponding concentrations from the calibration curve.
- The concentration read from the calibration curve must be multiplied by the respective dilution factor to obtain the effective clenbuterol concentration in samples expressed in ppb (μg/Kg or μg/l).

The dilution factors are:
urine samples (method I): 5
urine samples (method II): 1
liver samples (method I): 8
liver samples (method II): 2
muscle samples: 2,5
eye samples: 5
feed and milk powder samples: 100
hair samples: 5

Please note: For results calculation, Excel spreadsheets are available on website and can be downloaded directly from the bottom of the product page.

10 EXAMPLE OF CALIBRATION CURVE



11 RESULTS EVALUATION

After results elaboration, it is necessary to verify the assay performance. The verification is performed by comparison of obtained data with those given in kit specifications. If the values are out from the specifications given, it is advised to check the expiry date of the kit, the wavelength of absorbance filter, as well as the procedure employed. If no errors are found, contact our technical assistance.
In order to avoid false non-compliant results, it is necessary to adopt a decision limit (CCα). It is suggested to determine a decision limit for each matrix in your laboratory. In alternative, contact the technical assistance.
WARNING: substitution will be possible just in case of rendered kit. The kit must be stored in its integral version and at the temperature indicated in this booklet.

12 KIT SPECIFICATIONS

12.1 Assay specifications

Mean Bo absorbance	$\geq$ 0.7 OD_{450nm}
B/Bo 50%	0.1 – 0.5 ng/ml
Std duplicates mean C.V. (%)	$\leq$ 6 %

12.2 Assay performance

The kit performances hereby presented are from an in-house validation; Detection Capability (CCβ) was calculated as requested by of EU Decision 657/2002. A Perfomance Data Sheet (PDS) with more detailed informations is available upon request.

DETECTION CAPABILITY (CCβ)

MATRICE	ANALYTE (ppb)		
	clenbuterol	salbutamol	terbutaline
Urine-method I	1	3	
Urine-method II			1
Liver-method I	0.5	3	
Liver method II			1
Feed	15	60	
Milk powder	10	10	
Hair	2		

13 LIABILITY

Samples evaluated as positive using the kit have to be re-tested with a confirmation method.
Tecna 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.
Tecna shall not be liable for the unsafe use of the kit out of the current European safety regulations.

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