β-AGONIST FAST ELISA

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A microtiter plate based competitive enzyme immunoassay for screening and quantitative analysis on the presence of a wide variety of Beta-Agonists

EUROPROXIMA β-AGONIST FAST ELISA

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BRIEF INFORMATION

The β-Agonist FAST ELISA is a competitive enzyme immunoassay for measurement of the concentration of β-Agonists. With this ELISA-kit 96 analyses can be performed. Samples and standards are measured in duplicate which means that a total of 40 samples can be analyzed.
The ELISA kit contains all reagents to perform the assay. Reagents for sample preparation are not included in the kit.

1. INTRODUCTION

β-Agonistic drugs are used in human healthcare for the treatment of for instance asthma. In recent years, it has been established that a number of β-agonistic drugs may have repartitioning effects in meat producing animals. The flow of nutrients is apparently shifted from adipose tissue towards muscle tissue. The result is an improved lean meat deposition and higher production efficiency.
The use of β-agonists as feed additives is not permitted in the European Community. Nevertheless, there have been reported several incidences of the use of clenbuterol, salbutamol, mabuterol, mapenterol and bromobuterol in a number of European countries.
Urine is still the most frequently analysed sample material, however, other sample materials are used for different reasons. In farmhouses, urine, faeces, hair and feed can be sampled. Sampling of faeces is much easier and faster than sampling of urine, and the residue levels for β-agonists are comparable. At slaughter, edible tissues (liver, kidney and muscle) can be sampled next to body fluids (plasma, urine and bile) and eye samples. Bile is one of the most suitable sample material for the control on misuse of anabolic steroids and can be preferred for the control of both steroids and β-agonists. Plasma samples are frequently used for pharmacokinetic studies. Clenbuterol accumulates in the choroid/pigmented retinal epithelium tissue of the bovine eye and even after a withdrawal period of 140 days, clenbuterol can still be detected which makes the material extremely suitable for the control on misuse of clenbuterol. Clenbuterol is permitted for use as a drug in pregnant cows. For this reason a method for screening for the presence of clenbuterol in milk samples is included in this manual as well. A number of studies have been published using the β-agonist-ELISA kit of EuroProxima .

2. PRINCIPLE OF THE β-AGONIST FAST ELISA

The microtiter plate based β-agonist-ELISA consists of one precoated plate (12 strips, 8 wells each). Horseradish peroxidase (-HRP) labeled clenbuterol and clenbuterol standard solution or sample are added to wells. Free based β-agonists from the samples or standards and clenbuterol-HRP conjugate compete for the specific antibody binding sites (competitive enzyme immunoassay).
After an incubation step of 30 minutes, the non-bound reagents are removed in a washing step. The amount of bound clenbuterol-HRP conjugate is visualized by the addition of a substrate/chromogen solution (H_2O_2/TMB). Bound clenbuterol-HRP conjugate transforms the colourless chromogen into a coloured product.
The substrate reaction is stopped by the addition of sulfuric acid. The colour intensity is measured photometrically at 450 nm. The optical density is inversely proportional to the β-agonists concentration in the sample.

3. SPECIFICITY AND SENSITIVITY

The β-agonist. ELISA utilizes antibodies raised in rabbits against protein conjugated clenbuterol.

Cross-reactivity:	Clenbuterol	100%	Carbuterol	40%
	Salbutamol	90%	Mapenterol	35%
	Cimbuterol	90%	Cimaterol	20%
	Bromobuterol	80%		
	Mabuterol	60%		
	Terbutaline	45%		

The cross-reactivities are determined in a buffer system. The reported values may be different in samples due to matrix effects.
The test cannot discriminate between analytes and cross-reactive substances.

The Limit of detection (LOD) is determined under optimal conditions. Cut-off criteria need critical consideration.

Matrix	**Procedure**	**LOD (ppb)**
Milk	8.8.1	0.1
Milk	8.8.2	0.04
Tissue Liquid	8.5	0.4
Liver/Tissue	8.2.1	0.1
Liver/Tissue	8.2.2	0.12
Feed	8.6	1.0
Urine	8.1	0.2
Urine	8.2.1	0.15
Faeces/Kidney	8.3	0.25

If the sample is found to be non-compliant, the results shall be verified by re-analysis of the sample using a confirmatory method.

4. HANDLING AND STORAGE

- Kit and kit components should be stored at 2°C to 8°C in a dark place. For repeated use store kit components as specified under chapter 9.
- After the expiry date of the kit and/or components has passed, no further quality guarantee is valid.
- Bring all kit components including the microtiter plate to ambient (room) temperature before use.
- Dilute the kit components immediately before use, but after the components are brought to ambient temperature.
- Avoid condensation in the wells of the plate. Bring the sealed plate to ambient temperature before opening the plate sealing.
- The substrate chromogen solution can be stored in a refrigerator (2ºC to 8ºC) until the expiry date stated on the label.
- Exposure of the chromogen solution to light should be avoided.

Degeneration of the reagents may have occurred when the following phenomena are observed:

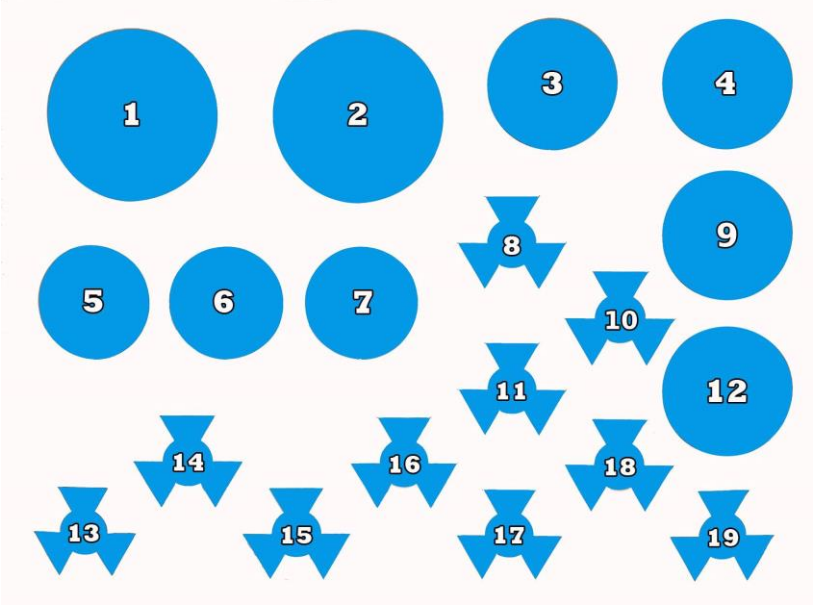
- A blue colouring of the chromogen solution before transferring it into the wells.
- A weak or no colour reaction in the zero standard wells (E450nm < 0.8).

5. KIT CONTENTS

Manual

One sealed (96-wells) microtiter plate (12 strips, 8 wells each), coated with antibody. Plate is ready-to-use.

Position of the reagents in the kit. For preparation of the reagents see Chapter 9.



1. **Dilution buffer** (20 ml, 4x concentrated)
2. **Rinsing Buffer** (30 ml, 20x concentrated)
3. **Substrate solution** (12 ml, Ready-to-use)
4. **Stop solution** (15 ml, Ready-to-use)
5. not in use
6. not in use
7. not in use
8. **Conjugate (**100x concentrated), blue cap)
9. not in use
10. not in use
11. **Standard solution 100 ng/ml**
12. not in use
13. **Zero Standard solution**(2ml, Ready-to-use)
14. **Standard solution 1** (1ml, Ready-to-use) **0.063 ng/ml**
15. **Standard solution 2** (1ml, Ready-to-use) **0.125 ng/ml**
16. **Standard solution 3** (1ml, Ready-to-use) **0.25 ng/ml**
17. **Standard solution 4** (1ml, Ready-to-use) **0.5 ng/ml**
18. **Standard solution 5** (1ml, Ready-to-use) **1.0 ng/ml**
19. **Standard solution 6** (1ml, Ready-to-use) **2.0 ng/ml**

6. EQUIPMENT AND MATERIALS REQUIRED BUT NOT PROVIDED

6.1 General equipment

- Gloves
- Fume hood
- Homogeniser (vortex, mixer)
- Automated microtiter plate washer or 8-channel micropipette 100 – 300 µl
- Microtiter plate shaker
- Microtiter plate reader with 450 nm filter
- Micropipettes, 10 – 100 µl
- Micropipettes, 100 – 1000 µl
- Multipipette with 2.5 ml combitips
- Centrifuge (4000 x g)
- Rotation mixer

6.2 Materials and chemicals

The materials and chemicals required for each sample preparation are indicated per chapter (starting chapter 8.1).

7. PRECAUTIONS

- This kit may contain hazardous substances. For hazard notes please refer to the appropriate safety data sheets (SDS).
- Avoid contact of all biological materials with skin and mucous membranes.
- Do not pipette by mouth.
- Do not eat, drink, smoke, store or prepare foods, or apply cosmetics within the designated work area.
- Do not use components past expiration date and do not use components from different lots.
- Each well is ultimately used as an optical cuvette. Therefore, do not touch the under surface of the wells, prevent damage and dirt.
- All components should be completely dissolved before use. Take special attention to the substrate and rinsing buffer, which crystallize at +4ºC.
- Optimal results will be obtained by strict adherence to this protocol. Careful pipetting and washing throughout this procedure are necessary to maintain good precision and accuracy.

8. SAMPLE PREPARATION

For each matrix as mentioned in the Introduction section a detailed method is given below.

8.1 Urine samples

Materials and chemicals required:

- Sample dilution buffer. See chapter 9

Urine samples can be applied directly after a 1:5 dilution in sample dilution buffer. Alternatively, to lower background of the urine samples, extraction procedures such as a Solid Phase Extraction (SPE) can be used.

Direct method for urine
Urine samples can be applied directly after a 1:5 dilution in sample dilution buffer.

- All samples are centrifuged for 5 minutes at 3000 x g.
- Pipette 50 µl of the supernatant into a glass tube, add 200 µl of sample dilution buffer and mix thoroughly (1:5 diluted samples).
- Use 25 µl of diluted sample in the ELISA.

To extend the measuring range samples can be diluted up to 1:20.

8.2.1. Urine, serum and liver samples SPE extraction procedure

Materials and chemicals required:

- Filter paper
- Sample dilution buffer. See chapter 9
- *Helix pomatia* juice (Merck 4114)
- 0,1 M HCl
- 1 M Tris base (Sigma T1503)
- Methanol 100%
- 0,1 M acetic acid
- 2% ammonium hydroxide (ammonia) solution :
  Add 6.25 ml of a 32% ammonia solution (Merck 5426) to 93,75 ml of 100% methanol.
- Agilent Technologies MP1-30 mg (A5321130) or UCT (UCT CSDAUA83) 80 mg DAU/3ml.

REMARK: In urine, serum and liver most of the hydroxyl group(s) containing β-agonists (such as salbutamol, terbutaline and carbuterol) are present as glucuronide and/or as sulphate. For the direct method, enzymatic hydrolysis (using *Helix pomatia* juice) is not necessary. However, applying extraction methods, enzymatic hydrolysis prior to the extraction of urine samples is recommended for an effective extraction of the hydroxyl group(s) containing β-agonists.

- Pipette 2 ml urine, serum or transfer 2 g thoroughly homogenized liver sample into a clean tube
- Pipette 20 µl (1:10 diluted in distilled water) *Helix pomatia* juice
- Add 7 ml 0.1 M HCl, vortex
- Incubate for 2 hours at 50ºC
- Mix head over head, 10 minutes (Rotor)
- Add 1 ml 1.0 M Tris base
- Mix head over head, 10 minutes (Rotor)
- Centrifuge the mixture, 10 minutes 2000 x g
- Take the upper layer and filtrate through a filter paper
- Enter the SPE procedure

Activation of the cartridge:

- Add 300 µl 100% methanol
- Add 300 µl 0.1 M acetic acid

Note: It is important that the cartridge is not allowed to dry up completely during activation and prior to sample addition! If the cartridge has become dry, repeat the activation procedure.

Carefully bring the 3 ml urine or serum filtrate onto the activated cartridge (flow 1 ml/min).

Note: For liver take 5 ml filtrate instead of 3 ml.

Elution of the β-agonists
- Pipette 1 ml 2% ammonium hydroxide solution in methanol on the cartridge
- Collect eluent
- Let the cartridges run dry for two minutes
- Evaporate the eluent under a mild stream of nitrogen at 50ºC
- Dissolve the residue in 0.6 ml dilution buffer and mix. N.B. For liver dissolve the residue in 2 ml sample dilution buffer and mix.
- Use 25 µl of the sample in the ELISA.

8.2.2. Procedure alternative method liver and tissue

- Weigh 2 gram homogenized liver sample in a clean tube.
- Add 8 ml of acetonitril and mix vigorously on a vortex mixer.
- Mix head over head for 10 minutes.
- Centrifuge 10 minutes at 2000xg..
- Pipette 650 µl from the supernatant into a glass tube.
- The acetonitril is evaporated to dryness under a mild stream of nitrogen at 50°C.
- The residue is dissolved in 250 µl dilution buffer, vortex vigorously.
- An aliquot of 25 µl is used in the ELISA test

8.3 Faeces and kidney

Materials and chemicals required:

- Sample dilution buffer. See chapter 9.
- 1 M NaOH
- 0.1 M HCl
- 0.1 M Acetic acid
- 1 M sodium carbonate (Merck 6392) pH 9.8
- Isobutanol (Merck 984)
- *Helix pomatia* juice (Merck 4114)
- 0.2 M sodium acetate buffer pH 4.8: dissolve 16.4 gram sodium acetate (Merck 6268) in 1 litre of distilled water. Check the pH and, if necessary, adjust the pH to 4.8 ± 0.1 with a few drops of 1 M NaOH.

Remark: β-agonists are mainly present as glucuronidated compounds. For the determination of β-agonists in combination with extraction procedures, enzymatic hydrolysis is necessary.

- Weight 1 g of homogenized faeces or kidney sample.
- Add 5 ml of 0.1 M HCL.
- Vortex for 1 min.
- Centrifuge for 10 min. at 4000 x g at +4°C.
- Transfer 2 ml of the supernatant (approximately 0.33 g of sample) into another tube.
- Add 2 ml of 0.2 M sodium acetate buffer pH 4.8.
- Add 20 µl of *Helix pomatia* juice and incubate overnight at 37°C or during 2 hours at 55°C.
- After this incubation, the pH of the sample has to be adjusted to 9.8 ± 0.2 by adding 0.25 ml of 1 M sodium carbonate pH 9.8 and, if necessary, a few drops of 1 M NaOH.
- Add 4 ml of isobutanol
- Vortex during 30 seconds

- Centrifuge for 5 min. at 2000 x g.
- Transfer 2 ml of the upper layer (isobutanol) into a glass tube.
- Evaporate to dryness under a mild stream of nitrogen at 50°C.
- Dissolve the residue in 500 µl of sample dilution buffer.
- Vortex during 30 seconds (0.33 g/ml of sample/ml buffer).
- Use 25 µl of the sample in the ELISA.

8.4 Choroid/retina

Materials and chemicals required:

- Sample dilution buffer. See chapter 9.
- 10 M NaOH
- Isobutanol (Merck 984)
- 0.1 M Tris-HCL buffer pH 8.0: dissolve 24.1 g Tris (Sigma T1503) and 19.47 g $CaCl_2.2H_2O$ (Sigma C8106) in 1 litre of distilled water. Adjust pH to 8.0 by adding concentrated HCl.
- Pronase E from *Streptomyces griseus* (Sigma P5147).
- 0.2 M sodium acetate buffer pH 4.8: dissolve 16.4 gram sodium acetate (Merck 6268) in 1 litre of distilled water. Check the pH and, if necessary, adjust the pH to 4.8 ± 0.1 with a few drops of 1 M NaOH.

- Remove the tissue from top of the eyeball.
- Cut the eyeball open at the side of the lens.
- Remove the vitreous humour and fold the eye inside out.
- Collect the choroid and retina in a glass tube.
- The previously weighed glass tube is weighed again. The difference in weight is the weight of the choroid/retina tissue.
- Add 4 ml of 0.1 M Tris-HCl buffer pH 8.0 containing 1.25 mg of pronase E.
- Incubate overnight at 55°C.
- Centrifuge for 10 min. at 1,500 x g.
- Pipette 2 ml of the supernatant into a test tube and adjust the pH to 9.4 ± 0.2 by adding a few drops of 10 M NaOH.
- Add 4 ml of isobutanol.
- Vortex for 30 sec.
- Wait for approximately 5 min. to allow a phase separation to occur.
- Pipette 2 ml of the upper layer (isobutanol) into a glass tube.
- Evaporate to dryness under a mild stream of nitrogen at 60°C.
- Dissolve the residue in 1 ml of sample dilution buffer. If the weight of the choroid/retina sample is 1 g, the dissolved residue contains 0.2 g sample per ml.
- Use 25 µl of the sample in the ELISA.

8.5 Tissue

Materials and chemicals required:

- Filter paper
- Sample dilution buffer. See chapter 9.
- 10 M NaOH
- 0.1 M HCl
- 0.1 M acetic acid
- Methanol 100%
- Isobutanol (Merck 984)
- 0.1 M Tris-HCL buffer pH 8.0: dissolve 24.1 g Tris (Sigma T1503) and 19.47 g $CaCl_2.2H_2O$ (Sigma C8106) in 1 litre of distilled water. Adjust pH to 8.0 by adding concentrated HCl.
- Pronase E from *Streptomyces griseus* (Sigma P5147).
- 1 M Tris base (Sigma T1503)
- 0.2 M sodium acetate buffer pH 4.8: dissolve 16.4 gram sodium acetate (Merck 6268) in 1 litre of distilled water. Check the pH and, if necessary, adjust the pH to 4.8 ± 0.1 with a few drops of 1 M NaOH.
- 2% Ammonium hydroxide (ammonia) solution: add 6.25 ml of a 32% ammonia solution (Merck 5426) to 93.75 ml of 100% methanol.
- Agilent Technologies MP1-30 mg (A5321130) or UCT (UCT CSDAUA83) 80 mg DAU/3ml.

Liquid extraction method

- Homogenise approximately 10 g of tissue.
- Weight 1 g of the homogenised sample and transfer into a glass tube.
- Add 4 ml of 0.1 M Tris-HCl buffer pH 8.0 containing 1.25 mg pronase E.
- Incubate overnight at 55ºC and centrifuge for 10 min at 2000 x g.
- Pipette 2 ml of the supernatant into a test tube and adjust the pH to 9.4 ± 0.2 by adding a few drops of 10 M NaOH for adjust pH and check using pH paper.
- Add 4 ml of isobutanol.
- Vortex for 30 sec and centrifuge for 10 minutes at 2000 x g.
- Pipette 2 ml of the upper layer (isobutanol) into a glass tube.
- Evaporate the isobutanol to dryness at 60ºC under a mild stream of nitrogen.
- Dissolve the residue in 250 µl of sample dilution buffer (1 g sample/ml).
- Use 25 µl of the solution in the ELISA.

SPE extraction procedure for tissue
- Homogenize an appropriate amount of the sample in a blender
- Weigh 2 gram of the homogenised sample in a tube
- Add 7 ml 0.1 M HCl, vortex
- Mix head over head, 10 minutes (Rotor)
- Add 1 ml 1.0 M Tris base
- Mix head over head, 10 minutes (Rotor)
- Centrifuge the mixture, 10 minutes at 2000 x g
- Take the upper layer and filtrate through a filter paper
- Enter the SPE procedure

Activation of the cartridge:

- Add 300 µl 100% methanol
- Add 300 µl 0.1 M acetic acid

Note: It is important that the cartridge is not allowed to dry up completely during activation and prior to sample addition! If the cartridge has become dry, repeat the activation procedure.

Carefully bring 3 ml tissue filtrate onto the activated cartridge (flow 1 ml/min).

Elution of the β-agonists
- Pipette 1 ml 2% ammonium hydroxide solution in methanol on the cartridge
- Collect eluent
- Let the cartridges dry for two minutes
- Evaporate the eluent under a mild stream of nitrogen at 50ºC
- Dissolve the residue in 0.6 ml sample dilution buffer and mix.
- Use 25 µl of the sample in the ELISA

8.6 Feed samples

Materials and chemicals required:

- 1 M NaOH
- 1 M HCl

- Approximately 50-100 g of sample is ground and pulverised into a fine powder.
- To 2 g of the ground feed sample, 2 ml of 1M HCl and 18 ml of distilled water are added.
- Vortex 3 minutes.
- Shake for 15 minutes at room temperature and centrifuge for 20 min. at 2000 x g.

- Decant supernatant and add 1 ml of 1 M NaOH, check the pH (7.0 – 7.8) and adjust if necessary by a few drops of 1 M HCl.
- Vortex and centrifuge for 20 min. at 2000 x g.
- Use 25 ul of the supernatant in the ELISA.

8.7 Hair samples

Materials and chemicals required:

- Sample dilution buffer. See chapter 9.
- 5 M HCl
- Tertiair butyl methyl ether (T-BME) (Merck 101849)

- Weight 0.1 g of pre-washed hair.
- Add 2.5 ml of 5 M NaOH.
- Heat the mixture for 10 min. at 95°C in a water-bath.
- Cool to room temperature.
- Add 5 ml of tertiair butyl methyl ether (T-BME).
- Mix head over head for 30 min.
- Centrifuge for 5 min. at 2000 x g.
- Transfer 2.5 ml of the upper layer (T-BME), containing the extracted β-agonists, into another tube.
- Evaporate to dryness under a mild stream of nitrogen at 50°C.
- Dissolve the residue in 500 µl of sample dilution buffer.
- Use 25 µl of the sample in the ELISA.
  NB: This method is only suitable for the detection of clenbuterol, bromobuterol, mapenterol and mabuterol [8].

8.8 Milk samples

Milk samples can be analyzed after a dilution step. To skimmed milk powder water has to be added to obtain milk, i.e. to 10 g milk powder an amount of 60 ml of distilled water is added.

Note: For undiluted milk samples the matrix effect is a critical factor. To avoid fat residues in the sample, the manual should be strictly followed.
The pH of the sample is another potential pitfall. Sour milk disturbs the ELISA, neutralization of the pH is essential.

An alternative method is given in case one needs to measure in the lower concentration region.

Materials and chemicals required:

- Acetonitril (Baker 9017)

8.8.1 Procedure:
- Centrifuge cold milk samples for 15 min. at 2000 x g at +4°C.
- Remove the upper fat layer with a spatula
- To 100 µl of defatted milk/reconstituted milk add 100 µl of dilution buffer and mix thoroughly
- Use 25 µl of the mixture in the ELISA test

8.8.2 Procedure (Alternative method)
- Pipette 2 ml milk in a clean tube.
- Add 8 ml of acetonitril and mix vigorously on a vortex mixer.
- Mix head over head for 10 minutes.
- Allow a supernatant to develop.
- Pipette 2 ml from the supernatant into a glass tube.
- The acetonitril is evaporated to dryness under a mild stream of nitrogen at 50°C.
- The residue is dissolved in 250 µl dilution buffer, vortex vigorously.
- An aliquot of 25 µl is used in the ELISA test.

9. PREPARATION OF REAGENTS

Before beginning the test, the reagents should be brought up to ambient temperature. Any reagents not used should be put back into storage immediately at +2ºC to +8ºC. Prepare reagents fresh before use.

Microtiter plate
Return unused strips into the resealable bag with desiccant and store at +2ºC to +8ºC for use in subsequent assays. Retain also the strip holder.

Dilution buffer
The dilution buffer is 4 times concentrated. Before dilution (20 ml buffer + 60 ml distilled water) the concentrated buffer should be at room temperature and thoroughly mixed. Concentrated buffer can show precipitates of the contents. Mix well before dilution with distilled water. The diluted buffer can be stored at +2ºC to +8ºC.

Conjugate solution (150 µl)
The conjugate is delivered 100x concentrated. Spin down the conjugate in the vial by a short centrifugation step (1 min., 1000xg). Add 15 µl of the concentrated conjugate solution to 1485 µl dilution buffer. Per 2 x 8 wells 1500 µl is required. Store unused concentrated conjugate at +2ºC to +8ºC.

Rinsing buffer
The rinsing buffer is delivered 20x concentrated. Prepare dilutions freshly before use. Per strip 20 ml of diluted rinsing buffer is used (1 ml concentrated rinsing buffer + 19 ml distilled water).

Standard solution (100 ng/ml)
To prepare standards in the appropriate matrix or to prepare spikes use the standard solution containing 100 ng Clenbuterol per ml. Dilute the standard solution in the appropriate matrix to make a dilution range of 2, 1, 0.5, 0.25, 0.125, 0.063 ng/ml. Also the zero standard should be of the same matrix.

Substrate/chromogen solution
The substrate/chromogen solution (ready-to-use) tends to precipitate at +4ºC. Take care that this vial is at room temperature when used (keep in the dark) and mix the content before pipetting into the wells.

Sample dilution buffer
To prepare additional sample dilution buffer dissolve in 1 L of distilled water (pH 7.4)

1.15 g	Na_2HPO_4
0.2 g	KH_2PO_4
0.2 g	KCl
60.0 g	NaCl
0.5 ml	Tween 80
10.0 g	BSA (Sigma A-7030)

10. ASSAY PROCEDURE

Rinsing protocol

In ELISA's, between each immunological incubation step, unbound components have to be removed efficiently. This is reached by appropriate rinsing. It should be clear that each rinsing procedure must be carried out with care to guarantee good inter- and intra-assay results.
Basically, manual rinsing or rinsing with automatic plate wash equipment can be performed as follows:

Manual rinsing

1. Empty the contents of each well by turning the microtiter plate upside down and remove residual liquid by striking the plate against a paper towel.
2. Fill all the wells to the rims (300 µl) with rinsing solution.
3. This rinsing cycle (1 and 2) should be carried out 3 times.
4. Turn the plate upside down and empty the wells by a firm short vertical movement.
5. Place the inverted plate on absorbent paper towels and tap the plate firmly to remove residual rinsing solution from the wells.
6. Take care that none of the wells dry out before the next reagent is dispensed.

Rinsing with automatic microtiter plate wash equipment

When using automatic plate wash equipment, check that all wells can be aspirated completely, that the rinsing solution is nicely dispensed reaching the rim of each well during each rinsing cycle. The washer should be programmed to execute three rinsing cycles.

Assay Protocol

1. Prepare samples according to Chapter 8 and prepare reagents according to Chapter 9.

2. Pipette 100 µl of zero standard in duplicate (wells H1,H2, blank).
   Pipette 25 µl of the zero standard (Bmax) in duplicate (wells A1, A2).
   Pipette 25 µl of each of the standard solutions in duplicate (wells B1,2 to G1,2 i.e. 0.063, 0.125, 0.25, 0.5, 1 and 2 ng/ml).

3. Pipette 25 µl of each sample solution in duplicate into the remaining wells of the microtiter plate.

4. Pipette 75 µl of conjugate (Clenbuterol-HRP) to all wells, except H1 and H2.

5. Seal the microtiter plate and shake the plate for a few seconds on a microtiter plate shaker.

6. Incubate the plate for 30 minutes in the dark at room temperature (20ºC to 25ºC).

7. Discard the solution from the microtiter plate and wash 3 times with rinsing buffer.

8. Pipette 100 µl of substrate solution into each well.

9. Incubate 30 minutes in the dark at room temperature (20ºC to 25ºC).

10. Add 100 µl of stop solution to each well.

11. Read the absorbance values immediately at 450 nm.

11. INTERPRETATION OF RESULTS

Subtract the mean optical density (O.D.) of the wells H1 and H2 (Blank) from the individual O.D. of the wells containing the standards and the samples.

The O.D. values of the six standards and the samples (mean values of the duplicates) are divided by the mean O.D. value of the zero standard/ Bmax (wells A1 and A2) and multiplied by 100. The zero standard/ Bmax is thus made equal to 100% (maximal absorbance) and the other O.D. values are quoted in percentages of the maximal absorbance.

$$\frac{\text{O.D. standard (or sample)}}{\text{O.D. zero standard/ Bmax}} \times 100 = \text{percentage maximal absorbance}$$

Calibration curve:
The values (percentage maximal absorbance) calculated for the standards are plotted on the Y-axis versus the analyte equivalent concentration (ng/ml) on a logarithmic X-axis.

Alternative for calibration curve:
The value of absorption (logit) calculation of the standards are plotted on Y-axis versus the analyte equivalent concentration on a logarithmic X-axis.

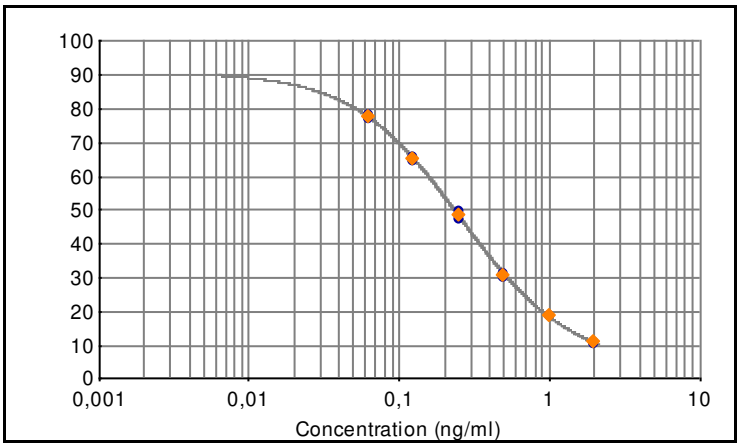


Figure 1 : Example of a calibration curve

The amount of β-agonist in the samples is expressed as β-agonist equivalents. The β-agonist equivalents in the samples (ng/ml) corresponding to the percentage maximal absorbance of each extract can be read from the calibration curve.

8.1 Urine samples (direct)

Applying the ELISA on urine samples, the calculated β-agonist equivalents have to be multiplied by a factor 5 (urine is 1:5 diluted in sample dilution buffer).

The multiplication factor is adapted according to the applied dilution of samples.

8.2.1. Urine and serum SPE extraction

The β-agonist equivalents measured in the extracts can be read directly from the calibration curve.

8.2.1. Liver samples (SPE extraction)

The β-agonist equivalents read from the calibration curve have to be multiplied by a factor 2.

8.2.2 Liver and tissue samples (alternative method)

The β-agonist equivalents found in the liver extracts have to be multiplied by a factor 1.92 to obtain ng/ml β-agonist equivalents in the sample.

8.3 Faeces and kidney

The β-agonist equivalents measured in the extracts have to be multiplied by a factor 3.

8.4 Choroid/retina

For choroid/retina, the calculated β-agonist equivalents have to be multiplied by a factor of 5 (the final extract contains 0.2 g of sample/ml) to obtain the β-agonist equivalent (ng/g) in the sample.

8.5 Tissue samples (liquid extraction method)

The calculated β-agonist equivalents in the extracts are equal to the β-agonist equivalents in muscle (1 g of sample/ml).

8.5 Tissue samples (SPE extraction method)

The calculated β-agonist equivalents can be read directly from the standard curve.

8.6 Feed samples

Applying the ELISA on feed samples, the calculated β-agonist equivalents have to be multiplied by a factor 11.

8.7 Hair samples

The β-agonist equivalents found in the hair extracts have to be multiplied by a factor 10 to obtain ng/g β-agonist equivalents in the sample.

8.8 Milk samples

8.8.1 Milk and milk powder samples

The β-agonist equivalents found in the milk extracts have to be multiplied by a factor 2 to obtain ng/ml β-agonist equivalents in the sample.

8.8.2 Milk samples (alternative method)

The β-agonist equivalents found in the milk extracts have to be multiplied by a factor 0.625 to obtain ng/ml β-agonist equivalents in the sample.

12. LITERATURE

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13. ORDERING INFORMATION

For ordering the β-Agonist FAST ELISA kit please use cat. code 5061BAGFc.

14. REVISION HISTORY

Textual changes in chapter 9.

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