CLENBUTEROL ELISA

5071BAGC[3]07.20

A competitive enzyme immunoassay for screening and quantitative analysis of Clenbuterol in various matrices

EUROPROXIMA CLENBUTEROL ELISA

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BRIEF INFORMATION

This Clenbuterol ELISA is a competitive enzyme immunoassay for the screening on the presence of β-agonists like clenbuterol, bromobuterol, mabuterol, mapenterol and cimbuterol in various matrices. The test is based on antibodies directed against clenbuterol. The ELISA kit contains a 96 microtiter plate as well as all essential reagents including ready-to-use standards to perform the test. Methods for a fast and efficient extraction of clenbuterol from different matrices are included in the kit manual.

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 toward 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, mabuterol, mapenterol and bromobuterol in a number of European countries. For the determination of misuse of β-agonists, analytical procedures based on HPLC or GC-MS have been reported. However, due to an extensive sample clean-up, such methods are expensive and time consuming.
Urine is still the most frequently analysed sample material, however, other sample materials are used for different reasons. In farmhouses, urine, faeces and feed can be sampled. Sampling of faeces is much easier and faster than sampling 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 materials for the control of 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. For all these sample materials described above, this protocol provides sample preparation procedures which can be used in combination with the Clenbuterol ELISA.

Two microtiter plate ELISA systems for the detection of β-agonists are available:

Clenbuterol ELISA (BAGC): This ELISA is based on clenbuterol antibodies and it is recommended for the detection of the following β-agonists: clenbuterol (100%), bromobuterol (100%), mapenterol (80%), mabuterol (70%) and cimbuterol (60%). The extraction procedures described in this manual are not recommended for the analysis of salbutamol (low recoveries).

β-Agonists ELISA (BAG): This ELISA is based on a mixture of antibodies raised against salbutamol and clenbuterol. Due to this antibody mixture, this generic test detects more
β-agonists than the Clenbuterol ELISA. This test is recommended for the detection of salbutamol (100%), clenbuterol (100%), bromobuterol (100%), cimbuterol (75%), mapenterol (70%), mabuterol (60%), tulobuterol (50%), clenpenterol (50%), terbutaline (40%), carbuterol (40%) and cimaterol (10%). The extraction procedures in this manual include an enzymatic hydrolysis step and are also suitable for salbutamol.

2. PRINCIPLE OF THE CLENBUTEROL ELISA

The kit is based on a microtiter plate (12 strips, each 8 wells), precoated with sheep antibodies to rabbit IgG. In one incubation step, specific antibody (rabbit anti-clenbuterol), enzyme labelled clenbuterol (enzyme conjugate) and clenbuterol standards or sample are added to the precoated wells. The specific antibodies are bound by the immobilised rabbit antibodies and at the same time free β-agonists (in the standard solution or in the sample) and enzyme labelled clenbuterol compete for the specific antibody binding sites (competitive enzyme immunoassay).
After an incubation time of one hour, the non-bound (enzyme labelled) reagents are removed in a washing step. The amount of bound enzyme conjugate is visualized by the addition of chromogen substrate (tetramethylbenzidine, TMB). Bound enzyme transforms the 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 and is inversely proportional to the β-agonists concentration in the sample.

3. SPECIFICITY AND SENSITIVITY

The Clenbuterol ELISA utilises a specific antibody raised in rabbits against clenbuterol.
The reactivity pattern of this antibody is:

Cross- reactions:	Clenbuterol	100 %
	Bromobuterol	100 %
	Mapenterol	80 %
	Mabuterol	70 %
	Cimbuterol	60 %
	Salbutamol	6 %
	Cimaterol	6 %
	Carbuterol	5 %
	Terbutaline	4 %
	Pirbuterol	4 %

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.

	Procedure	Clenbuterol (ppb)	Bromobuterol (ppb)	Mapenterol (ppb)	Mabuterol (ppb)	Cimbuterol (ppb)
Urine Direct	8.1.1	0.5	0.5	0.6	0.7	0.8
Urine liquid extraction	8.1.2	0.05	0.05	0.06	0.07	0.08
Feed	8.2	5	5	6	7	8
Tissue / liver	8.3	0.2	0.2	0.3	0.3	0.3
Choroid/ retina	8.4	0.5	0.5	0.6	0.7	0.8
Muscle	8.5	0.1	0.1	0.1	0.1	0.2

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:

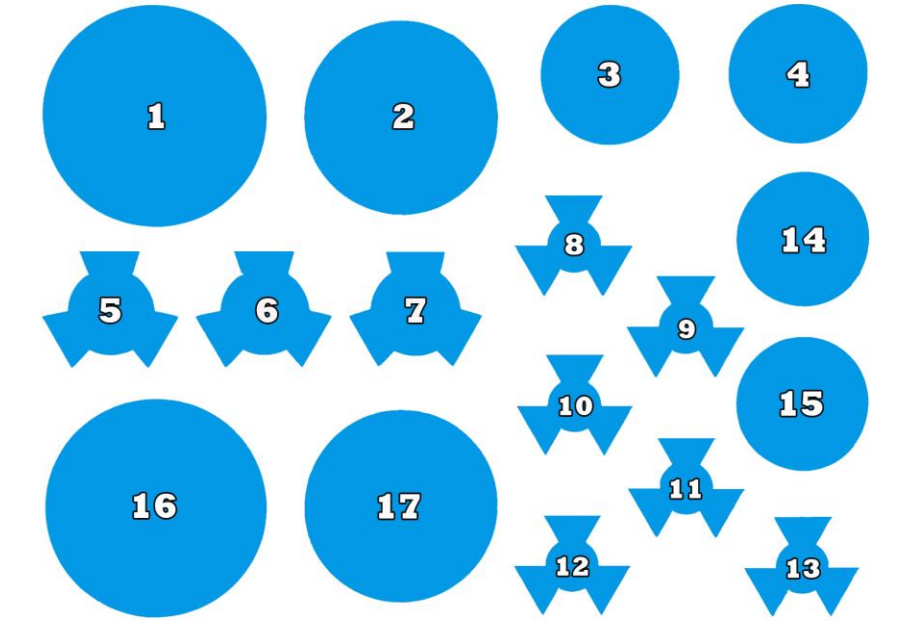
- A blue colouring of the chromogen solution before transferring it into the wells.
- A weak or no colour reaction in the zero standard wells (E450nm < 0.8).

5. KIT CONTENTS

Manual

One sealed microtiter plate (12 strips, 8 wells each), coated with antibodies to rabbit IgG. Ready-to-use.

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



1. **Dilution buffer**	(40 ml)		
2. **Rinsing buffer**	(30 ml, 20x concentrated)		
3. **Substrate solution**	(12 ml, Ready-to-use)		
4. **Stop solution**	(15 ml, Ready-to-use)		
5. **Conjugate**	(lyophilized, blue cap)		
6. **Antibody**	(lyophilized, yellow cap)		
7. **Zero standard**	(2 ml, Ready-to-use)		
8. **Standard solution 1**	(1ml, Ready-to-use)	**0.125**	**ng/ml**
9. **Standard solution 2**	(1ml, Ready-to-use)	**0.25**	**ng/ml**
10. **Standard solution 3**	(1ml, Ready-to-use)	**0.5**	**ng/ml**
11. **Standard solution 4**	(1ml, Ready-to-use)	**1.0**	**ng/ml**
12. **Standard solution 5**	(1ml, Ready-to-use)	**4.0**	**ng/ml**
13. **Standard solution 6**	(1ml, Ready-to-use)	**8.0**	**ng/ml**
14. **Standard solution 50 ng/ml**			
15. not in use			
16. not in use			
17. not in use			

6. EQUIPMENT AND MATERIALS REQUIRED BUT NOT PROVIDED

- Scales and weighing vessels
- Gloves
- Fume hood
- Homogeniser (vortex, mixer)
- Centrifuge (2000 x g)
- Automated microtiter plate washer or 8-channel micropipette 100 – 300 µl
- Microtiter plate shaker
- Microtiter plate reader with 450 nm filter
- Micropipettes, 100 – 1000 µl
- Multipipette with 2.5 ml combitips
- 4 ml glass tubes
- 15 ml tubes with screw cap (Greiner, polypropylene)
- NaOH 1M and 10M
- Tert. Butyl methyl ether
- Acetonitrile
- Methanol 100%
- HCl 1M
- Phosphoric acid
- Isobutanol
- Borax ($Na_2B_4O_7.10H_2O$)
- Boric acid (H_3BO_3)
- Tris
- Calcium Chloride
- Pronase E from Streptomyces griseus

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

8.1 Urine

8.1.1 Urine samples (direct method)
Urine samples can be applied directly.
- Use 50 µl of urine sample in the ELISA.

8.1.2 Extraction of urine samples

REMARK: This extraction procedure can be used to lower the background of urine samples.

- Add 1 ml of (0.1 M di-Sodium tetraborate, pH 8.0 (see chapter 9) to 1 ml of the urine sample
- Check the pH > 8.0 and if necessary, adjust with a few drops of 1 M NaOH
- Add 4 ml of tert.-Butylmethyl ether (T-BME)
- Vortex for 30 seconds
- Wait for approximately 5 minutes to obtain phase separation
- Pipette 2 ml of the upper layer (T-BME) into a glass tube
- Evaporate the T-BME to dryness at 50ºC under a mild stream of nitrogen
- Dissolve the residue in 250 µl of dilution buffer
- Use 50 µl of this solution in the ELISA test

8.2 Feed samples:
- Weigh 1 g of the feed sample and transfer in 50 ml vials
- Add 5 ml of 0.2 M phosphoric acid and 5 ml of methanol
- Extract under continuous movement for 30 minutes
- Centrifuge for 15 minutes, 2000 x g
- Pipette 0.125 ml of the supernatant into a clean tube and add 500 µl of dilution buffer (pH 7.4)
- Check the pH (7 ± 0.5) and if necessary, adjust the pH with a few drops of 1 M NaOH or 1 M HCl
- Centrifuge for 10 minutes, 2000 x g
- The supernatant should be clear, if not, use 0.45 µm filters
- Use 50 µl of this solution in the ELISA test.

8.3 Liver and tissue samples:
- Weigh 2 g of homogenized liver sample in a tube
- Add 8 ml of acetonitrile and mix vigorously on a vortex mixer
- Mix head over head, 10 minutes
- Centrifuge for 10 minutes, 2000 x g
- Pipette 650 µl of the supernatant into a glass-tube
- The acetonitril is evaporated to dryness under a mild stream of nitrogen at 50°C
- Dissolve the residue in 250 µl of dilution buffer, vortex vigorously
- Use of 50 µl of this solution in the ELISA test.

8.4 Choroid/retina

- Remove the 'tissue' from top of the eyeball. Cut open the eyeball 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 0.2 M Tris.HCl buffer pH 8.0 (see chapter 9) containing 1.25 mg pronase E.
- Incubate overnight at 55ºC
- Centrifuge (10 minutes, 2000 x g)
- Pipette 2 ml of the supernatant into a test tube
- Adjust the pH to 9.4 ± 0.2 by adding a few drops of 10 M NaOH
- Add 4 ml of isobutanol
- Vortexing for 30 seconds
- Wait for approximately 5 minutes to obtain phase separation
- Pipette 1 ml of the upper layer (isobutanol) into a glass tube
- Evaporate the isobutanol at 60ºC under a mild stream of nitrogen
- Dissolve the residue in 0.5 ml of dilution buffer
- Use 50 µl of this solution in the ELISA test.

N.B. If the weight of the choroid/retina sample is 1 g then the dissolved residue contains 0.25 g sample/ml.

8.5 Muscle samples

- Homogenise approximately 10 g of tissue
- Weight 1 g of the homogenised sample and transfer into a glass tube
- Add 4 ml of 0.2 M Tris.HCl buffer pH 8.0 (see chapter 9) containing 1.25 mg pronase E.
- Incubate overnight at 55ºC
- Centrifuge (10 minutes, 2000 x g)
- Pipette 2 ml of the supernatant into a test tube
- 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 seconds
- Wait for approximately 5 minutes to obtain phase separation
- Pipette 2 ml of the upper layer (isobutanol) into a glass tube
- Evaporate the isobutanol at 60ºC under a mild stream of nitrogen
- Dissolve the residue in 250 µl of dilution buffer
- Use 50 µl of this solution in the ELISA test.

8.6 Hair samples
- Weigh an amount of 0.1 g of pre-washed hair into a test-tube (9 x 1.4 cm)
- Add 2.5 ml of 5 M NaOH
- Heat the mixture for 10 minutes at 95ºC in a water-bath
- Cool to 20°C - 25°C and add 5 ml of tert-Butylmethyl ether (T-BME)
- Mix the content in the tubes for 30 minutes (head over head)
- Centrifuge the mixtures for 5 minutes, 2000 x g
- Transfer 2.5 ml of the upper layer (T-BME) into another tube
- Evaporate the T-BME to dryness at 50ºC under a mild stream of nitrogen
- Dissolve residue in 500 µl of dilution buffer
- Use 50 µl of this solution in the ELISA test.

9. PREPARATION OF REAGENTS

Before beginning the test, the reagents should be brought up to ambient temperature. Any reagent not used should be put back into storage immediately at 2°C to 8°C. 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.

Rinsing buffer
The rinsing buffer is delivered 20 times 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).

Substrate solution
The substrate solution (ready-to-use, 12 ml) precipitates at 4ºC. Take care that this vial is at 20°C - 25°C (keep in the dark) and mix the content before pipetting into the wells.

Clenbuterol Standard solution (50 ng/ml)
To prepare standards in the appropriate matrix or to prepare spikes use the standard solution containing 50 ng clenbuterol per ml. Dilute the standard solution in the appropriate matrix to make a dilution range of 8, 4, 1, 0.5, 0.25 and 0.125 ng/ml. Also the zero standard should be in the same matrix.

Conjugate solution
Reconstitute the vial of lyophilized conjugate (Clenbuterol-HRP) with 4 ml dilution buffer, mix thoroughly and keep in the dark until use. Store the vial immediately after use in the dark at 2ºC to 8ºC. For prolonged storage, prepare aliquots and store the aliquots at -20°C until expiration date.

Antibody solution
Reconstitute the vial of lyophilised antibodies with 4 ml dilution buffer, mix thoroughly and keep in the dark until use. Store the vial immediately after use in the dark at 2ºC to 8ºC. For prolonged storage, prepare aliquots and store the aliquots at -20°C until expiration date.

0.1 M di-Sodiumtetraborate pH 8.5
A) Dissolve 9.54 $Na_2B_40_7.10H_20$ (Borax) in 250 ml aqua dest (Mw 381.37).
B) Dissolve 3.09 g H_3BO_3 in 400 ml aqua dest (Mw 61.83).
Add A to B till pH is 8.5

0.2 M Tris.HCl
24.2 g Tris and 14.7 g $CaCl_2$ in 1 litre of distilled water
Adjust pH to 8 with 1 M HCl

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 washing 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 rim (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 washing solution in the wells.
6. Take care that none of the wells dry out before the next reagent is dispensed.

Rinsing with automatic microtiter plate washing 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 (blank; well H1, H2).
   Pipette 50 µl of zero standard in duplicate (zero standard; well A1, A2).

3. Pipette 50 µl of each standard dilution in duplicate (well B1,2 to G1,2).

4. Pipette 50 µl of each sample solution in duplicate into the remaining wells of the microtiter plate.

5. Add 25 µl of conjugate (Clenbuterol-HRP) to all wells, except wells H1 and H2.

6. Add 25 µl of antibody solution to all wells, except wells H1 and H2.

7. Seal the microtiter plate and shake the plate for 1 minute.

8. Incubate for 30 minutes in the dark at 20ºC – 25ºC.

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

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

11. Incubate 15 minutes at 20ºC - 25ºC.

12. Add 100 µl stop solution to each well.

13. Read the optical density (OD) 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 (% 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 absorption value of the standards is plotted on the Y-axis versus the concentration on the X-axis. The Y-axis is in logit the Y-axis is logarithmic.

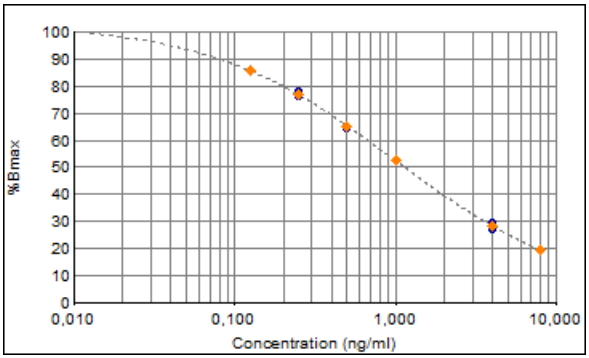


Figure 1 : Example of a calibration curve

The amount of ß-Agonist in the samples is expressed as clenbuterol equivalents. The clenbuterol equivalents in the urine and extracts correspond to the percentage maximal absorbance of each urine or extract read from the calibration curve.

8.1.1 Urine samples (direct)
The calculated Clenbuterol equivalents are directly read from the standard curve and match the clenbuterol content in the urine sample.

8.1.2 Urine samples (liquid extraction)
The clenbuterol equivalents, as read from the standard curve, have to be divided by a factor 2 to obtain the clenbuterol content in the urine sample.

8.2 Feed samples
The clenbuterol equivalents, as read from the standard curve, have to be multiplied by a factor of 50 to obtain the clenbuterol content in the feed sample.

8.3 Liver and tissue samples
The clenbuterol equivalents, as read from the standard curve, have to be multiplied by a factor of 1.92 to obtain the clenbuterol content in the sample.

8.4 Choroid/retina samples
The clenbuterol equivalents, as read from the standard curve, have to be multiplied by a factor of 4 (assumed the final extract contains 0.25 g of sample/ml) to obtain the clenbuterol content in the sample.

8.5 Muscle samples
For muscle, the calculated clenbuterol equivalents in the extracts are equal to the clenbuterol eguivalents in muscle.

8.6 Hair samples
The clenbuterol equivalents, as read from the standard curve, have to be multiplied by a factor 10 to obtain the clenbuterol content in hair samples.

12. LITERATURE

Courtheyn, D., Desaever, C., Verhe, R., High-performance liquid chromatographic determination of clenbuterol and cimaterol using post-column derivatization. J. Chromatogr., 564, 1991, 537.
Schilt, R., Haasnoot, W., Jonker, M.A., Hooijerink, H., Paulussen, R.J.A., Determination of β-agonistic drugs in feed, urine and tissue samples of cattle with immuno-affinity chromatography and GC-MS. In Proceedings of the Euro Residue Conference, ed. N. Haagsma, A. Ruiter and P.B. Czedik-Eysenberg, Rijksuniversiteit Utrecht, Faculteit der Diergeneeskunde, Utrecht, 1990, 320.
Haasnoot, W., Cazemier, G., Stouten, P. and Kemmers-Voncken, A. Immunochemical Approaches to the Analysis of ß-Agonistic Drugs. ACS-Books, Residue Analysis in Food Safety: Applications of Immunoassay Methods. Eds. Ross. C. Beier and Larry H. Stanker 1996.
Elliott C.T., Crooks, S.R.H., McEvoy, J.G.D., McCaughey, W.J., Hewitt, S.A., Patterson, D. and Kilpatrick, D. Vet. Res. Commun. 17, 1993, 459.
Haasnoot, W., Stouten, P., Schilt, R., Hooijerink, D. A fast immunoassay for the screening of β-agonists in hair. Analyst 123, (1998), 2707.

13. ORDERING INFORMATION

For ordering the Clenbuterol ELISA kit please use cat. code 5071BAGC.

14. REVISION HISTORY

The manual is adapted to a new layout of the test kit. Several textual changes are added.

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