β-AGONIST ELISA

A microtiter plate based competitive enzyme immunoassay for analysis and screening of urine, faeces, feed, bile, tissue, plasma, hair and choroid/retina samples

β-AGONIST-ELISA

A microtiter plate based competitive enzyme immunoassay for analysis and screening of urine, faeces, feed, bile, tissue, plasma, hair and choroid/retina samples on the presence of:

clenbuterol	tulobuterol
salbutamol	clenpenterol
bromobuterol	clenproperol
cimbuterol	terbutaline
mapenterol	carbuterol
mabuterol	cimaterol

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BRIEF INFORMATION

This β-agonist ELISA is a competitive enzyme immunoassay for the screening of urine, faeces, feed, bile, tissue, plasma and choroid/retina samples on the presence of several β-agonists. The test is based on a mixture of antibodies raised against salbutamol and clenbuterol and has a good sensitivity for β-agonistic drugs such as clenbuterol, salbutamol, bromobuterol, cimbuterol, mapenterol, mabuterol, tulobuterol, clenpenterol, clenproperol, terbutaline, carbuterol and cimaterol. With this ELISA-kit 96 analyses can be performed. Samples and standards are measured in duplicate which means that in total 40 samples can be analysed. The ELISA kit contains all the reagents, including standards, required to perform the test. Materials and chemicals necessary for extraction or concentration steps are not included in the test-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 [1]. 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 [7] 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 [3,4,5,6].

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 prescribed in the Clenbuterol-ELISA 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 (see Section 3) than the Clenbuterol ELISA. The extraction procedures in this manual include an enzymatic hydrolysis step and are also suitable for screening for salbutamol.

2. PRINCIPLE OF THE β-AGONIST-ELISA

The microtiter plate based ELISA kit consists of 12 strips, each containing 8 wells, precoated with sheep antibodies to rabbit IgG. Specific antibodies (rabbit anti-clenbuterol and anti-salbutamol), horseradish peroxidase labelled salbutamol (enzyme conjugate) and salbutamol standards or samples are added to the precoated wells followed by a single incubation step. The specific antibodies are bound by the immobilized rabbit antibodies and at the same time free β-agonists (in the standard solution or in the sample) and enzyme labelled salbutamol 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 substrate chromogen (tetramethylbenzidine, TMB). Bound enzyme transforms the chromogen into a coloured product.
The substrate reaction is stopped by the addition of sulphuric 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 β-agonist-ELISA utilises a mixture of antibodies raised in rabbits against clenbuterol and salbutamol. The reactivity pattern of these antibodies to the β-Agonist group was checked for the following compounds:

Cross- reactions:				
	Salbutamol	100%	Tulobuterol	50%
	Clenbuterol	100%	Clenpenterol	50%
	Bromobuterol	100%	Clenproperol	50%
	Cimbuterol	75%	Carbuterol	40%
	Mapenterol	70%	Terbutaline	40%
	Mabuterol	60%	Cimaterol	10%

11. LITERATURE

1. Haasnoot, W., Cazemier, G., Stouten, P. and Kemmers-Voncken, A. Immunochemical Approaches to the Analysis of β-Agonistic Drugs. ACS-Books, Residue Analysis in Food Safety: Applications of Immunoassay Methods. Eds. Ross. C. Beier and Larry H. Stanker (1996).
2. 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.
3. I. Nausch and K. Galley. Fast screening of β-agonist residues in eyes of meat stock by enzyme immunoassay (ELISA. Arch. für Lebensmittelhygiene, 50 (1999), 104-105.
4. I. Nausch and K. Galley. Current clean-up procedures for the determination of β-agonists in bovine urine by enzyme immunoassay (ELISA). Arch. für Lebensmittelhygiene, 48 (1997), 56-58.
5. J. Haines and P.D. Patel. A brief evaluation of the performance of three commercial ELISAs for the analysis of β-agonists in foods. The British Food Manufacturing Industries Research Association, 124, (1998), 1-11.
6. S. Hahnau and B. Julicher. Evaluation of commercially available ELISA testkits for the detection of clenbuterol and other β-agonists. Food Add. and Contam., 13, (1996), 259-274.
7. Central Laboratory of the National Inspection Service for Lifestock and Meat (CLRVV). Personal Communication (1997).
8. Haasnoot, W., Stouten, P., Schilt, R., Hooijerink, D. A fast immunoassay for the screening of β-agonists in hair. Analyst 123, (1998), 2707.

12. ORDERING INFORMATION

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

EuroProxima B.V.
Beijerinckweg 18
NL 6827 BN Arnhem
The Netherlands

TEL: + 31 26 3630364
FAX: + 31 26 3645111
Web-site:http://www.europroxima.com
E-mail: info@europroxima.com

Muscle samples (liquid extraction method)
The calculated salbutamol equivalents in the extracts are equal to the salbutamol equivalents in muscle (1 g of sample/ml).

Muscle samples (SPE extraction method)
The calculated salbutamol equivalents read from the standard curve have to be multiplied by a factor 2,5.

Feed samples
Applying the ELISA on feed samples, the calculated salbutamol equivalents have to be multiplied by a factor 50 (0.02 g feed samples/ml buffer).

Hair samples

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

Milk samples
The salbutamol equivalents found in the milk samples have to be divided by a factor 2 to obtain ng/g salbutamol equivalents in the milk sample.

Skimmed milk powder
The salbutamol equivalents found in the solubilised milk powder samples have to be multiplied by a factor 3.5 to obtain ng/g salbutamol equivalents in the milk powder sample.

The detection limits of the test in ppb are:

	Urine Direct	Urine Liquid-extraction	Faeces, liver, kidney bile, plasma	Muscle SPE extr.	Retina	Feed	Milk
Salbutamol	0.75	0.1	0.25	0.2	0.8	10	--
Clenbuterol	0.75	0.1	0.25	0.2	0.8	10	0.03
Bromobuterol	0.75	0.1	0.25	0.2	0.8	10	0.03
Cimbuterol	1.3	0.15	0.35	0.3	1.1	13	--
Mapenterol	1.3	0.15	0.35	0.3	1.1	13	--
Mabuterol	1.7	0.15	0.4	0.3	1.3	17	--
Tulobuterol	2	0.2	0.5	0.4	1.6	20	--
Clenpenterol	2	0.2	0.5	0.4	1.6	20	--
Clenproperol	2	0.2	0.5	0.4	1.6	20	--
Carbuterol	2.5	0.25	0.6	0.5	2.0	25	--
Terbutaline	2.5	0.25	0.6	0.5	2.0	25	--
Cimaterol	10	1.0	2.5	2.0	8.0	100	--

4. HANDLING AND STORAGE

- Store the kit at +2ºC to +8ºC in a dark place.
- After the expiry date (see kit label) has passed, it is no longer possible to accept any further quality guarantee.
- Avoid condensation in the ELISA plate after the plate is transported from the refrigerator to room temperature. Before opening the sealed plate, the plate should be at ambient temperature.
- Reconstitute or dilute the kit components immediately before use, but after the components are at ambient temperature.
- After the lyophilised kit components have been reconstituted, these components have to be used directly or can be stored in a refrigerator for maximally one week (stored at +2ºC to +8ºC in the dark). When no refrigerator (+2ºC to +8ºC) is available or when the antibody and conjugate components have to be stored for more than one week, these components should be stored in aliquots at –20ºC immediately after the first use. Alternatively, after reconstitution of the antibody and conjugate components, aliquots of these solutions can be prepared. The aliquots can be stored in a freezer (–20ºC) for at least one year. The substrate and standard solutions can be stored in a refrigerator (+2ºC to +8ºC) until the expiry date stated on the label.
- Any direct action of light on the substrate/chromogen solution should be avoided.

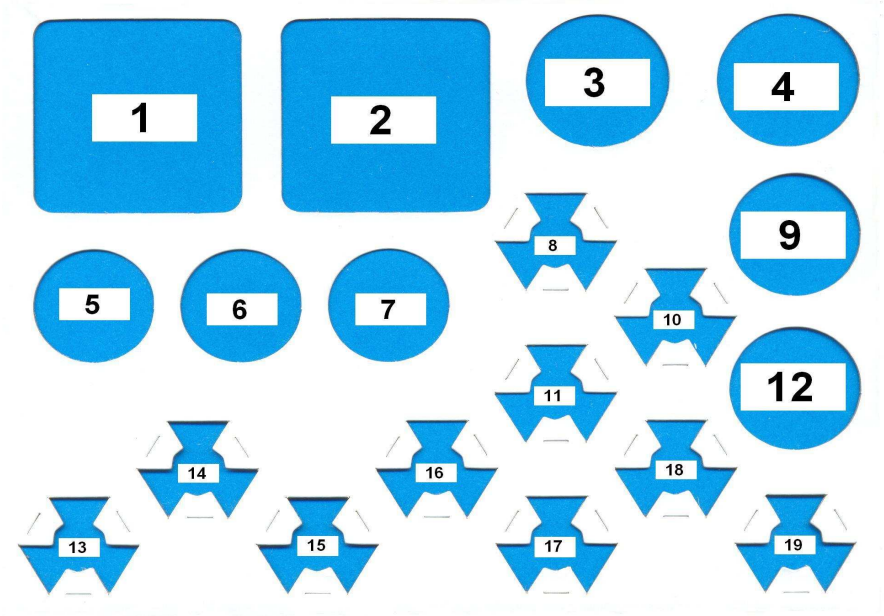
If the following phenomena are observed, this may indicate a degeneration of the reagents:
- A blue colouring of the substrate/chromogen solution before putting it into the wells,
- A weak or absent colour reaction of the first standard (zero standard) (E450nm < 0.8).

5. KIT CONTENTS

Manual

1 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 8.



1. **Dilution buffer** (20 ml, Ready to use)
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** (lyophilised, blue cap)
6. **Antibody** (lyophilised, yellow cap)
7. not in use
8. **Standard 100 ng/ml** (1.0 ml)
9. not in use
10. not in use
11. not in use
12. not in use
13. **Zero Standard** (2 ml, Ready to use)
14. **Standard solution 1** (1ml, Ready to use) **0.062 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**

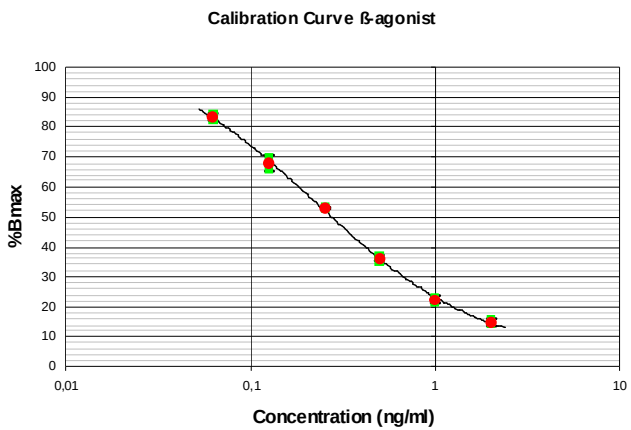


Figure 1 : Example of a calibration curve

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

Urine samples (direct)
Applying the ELISA on urine samples, the calculated salbutamol equivalents have to be multiplied by a factor 5 (urine is 5 x diluted in sample dilution buffer).

Urine samples (liquid-liquid extraction)
The salbutamol equivalents are equal to the concentration in urine (dilution factor 1).

Urine samples (solid phase extraction)
The salbutamol equivalents have to be divided by a factor 4 (4x concentration of the urine).

Faeces, liver, kidney
The salbutamol equivalents measured in the extracts have to be multiplied by a factor 2.5 (0.40 g sample/ml buffer).

Bile and plasma
The salbutamol equivalent in the extracts have to be multiplied by a factor 2.5 (0.4 ml sample/ml extract).

Choroid/retina
For choroid/retina, the calculated salbutamol equivalents have to be multiplied by a factor of 4 (the final extract contains 0.25 g of sample/ml) to obtain the salbutamol equivalent (ng/g) in the sample.

Assay Protocol

1. Prepare samples according to chapter 7 (Sample treatment) and prepare reagents according to chapter 8.

2. Pipette 100 µl of zero standard in duplicate (well A1, A2).
   Pipette 50 µl of zero standard in duplicate (well B1, B2).
   Pipette 50 µl of each standard dilution in duplicate (well C1,2 to H1,2, i.e. 0.062, 0.125, 0.25, 0.5, 1.0 and 2.0 ng/ml).
   Pipette 50 µl of each sample solution in duplicate into the remaining wells of the microtiter plate (40 samples; 80 wells).

3. Add 25 µl of conjugate (SB-HRPO) to all wells, except wells A1 and A2.

4. Add 25 µl of antibody solution to all wells, except wells A1 and A2.

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

6. Incubate for 1 hour in the dark at 4ºC. (2ºC - 8ºC)

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

8. Pipette 100 µl substrate solution into each well. Incubate 30 min. at room temperature (20ºC - 25ºC).

9. Add 100 µl stop solution to each well.

10. Read the absorbance values immediately at 450 nm.

10. INTERPRETATION OF RESULTS

Subtract the mean optical density (O.D.) of the wells A1 and A2 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 (wells B1 and B2) and multiplied by 100. The zero standard is thus made equal to 100% (maximal absorbance) and the other O.D. values are quoted in percentages of the maximal absorbance.

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

Calibration curve:
The values (% maximal absorbance) calculated for the standards are plotted (on the Y-axis) versus the salbutamol equivalent concentration (ng/ml) on a logarithmic X-axis. The calibration curve should be virtually linear in the 0.06 - 2 ng/ml range.

6. SAFETY PRECAUTIONS

- The stop reagent contains 0.5 M sulphuric acid. Do not allow the reagent to get into contact with the skin.
- 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.
- TMB is toxic by inhalation, in contact with skin and if swallowed; observe care when handling the substrate.
- Do not use components past expiry date and do not intermix components from different serial 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, which might crystallise at 4ºC.
- Optimal results will be obtained by strict adherence to this protocol. Careful pipetting and washing throughout this procedure are necessary to maintain precision and accuracy.

7. SAMPLE TREATMENT

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

7.1 Urine samples

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

7.1.1 **Direct method for urine**

Urine samples can be applied directly after a five times dilution in sample dilution buffer.

- All samples are centrifuged for 5 minutes at 2000 x g.
- Pipette 50 µl of the supernatant into a glass tube, add 200 µl of sample dilution buffer and mix thoroughly (5 times diluted samples).
- Use 50 µl of diluted sample in the ELISA.

7.1.2 **Extraction procedures for urine**

REMARK I: In urine, 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.

7.1.2.1 Hydrolysis of urine samples

- Add 2 ml of 0.2 M acetate buffer* (pH 4.8) to 2 ml of the urine sample
- Check the pH and if necessary adjust the pH to 4.8 with a few drops of 1 M acetic acid
- Add 20 µl of Helix pomatia Juice (Merck. art. no. 4114)
- Incubate overnight at 37ºC or during 2 hours at 55ºC
- Cool to room temperature before applying samples to one of the extraction procedures

REMARK II: Two extraction procedures are described. Both are suitable for the extraction of β-agonists from urine samples. Compared with the SPE procedure, the LLE procedure is easier to perform and results in higher recoveries for most of the β-agonists [1,7].

* Dissolve 16.4 g sodium acetate (Merck 6268) in bi-distilled water. Adjust pH to 4.8 with acetic acid.

7.1.2.2 Liquid-liquid Extraction (LLE) procedure

- After hydrolysis (7.1.2.1), add 2 ml of 0.25 M Sodium carbonate buffer pH 9.8
- Check and/or adjust pH (9.8 ± 0.2) with 1 M NaOH
- Add 2 ml of isobutanol to 1.5 ml of the diluted urine sample, mix (vortex) for 1 min and centrifuge (2-5 min at 2000 x g)
- Evaporate 1 ml of the upper layer (isobutanol) at 50ºC under a mild stream of nitrogen
- Dissolve the residue in 250 µl of sample dilution buffer (1 ml of sample/ml buffer)
- Pipette 50 µl in the microtiter plate.

7.1.2.3 Solid Phase Extraction (SPE) procedure

- After hydrolysis (7.1.2.1), add 2 ml of 0.1 M phosphate buffer (pH 6.0) and adjust the pH to 6.0 ± 1.0 by adding drops of 1 M NaOH.
- The urine samples are purified by means of Bond Elute Certify columns from Varian (3 ml columns, art. nr. 1210-2051) as follows:
- Activate the columns by washing successively with 2 ml of 100% methanol and with 2 ml of 0.1 M phosphate buffer (pH 6.0).
- Transfer the hydrolysed and pH 6.0 adjusted urine samples onto the activated columns and draw them slowly through the columns (it should at least take 2 min; do not dry the columns.)
- Wash the columns with 1 ml of 1.0 M acetic acid. Dry the column under vacuum for 5 min.
- Wash the column with 6 ml of 100% methanol. Dry the column under vacuum for 2 min.
- Elute the β-agonists with 5 ml of 2% ammonium hydroxide in ethyl acetate.
- Evaporate the eluent to dryness at 50ºC under a mild stream of nitrogen.
- Dissolve the residue in 500 µl of sample dilution buffer and pipette 2 x 50 µl in the test-kit (4 times concentrated samples).

Sample dilution buffer: Chapter 8, page 12

Conjugate solution

Reconstitute the vial of lyophilised conjugate (SB-HRPO) 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.

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.

Sample Dilution buffer

To prepare additional dilution buffer dissolve in 1 L of distilled water

1.15	g	Na_2HPO_4
0.2	g	KH_2PO_4
0.2	g	KCl
30.0	g	NaCl
0.5	ml	Tween 20

9. ASSAY PROCEDURE

Rinsing protocol

In ELISA's, between each immunological incubation step, un-bound 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 done as follows:

Manual rinsing

1. Empty the contents of each well by turning the microtiter plate upside down followed by a firm short vertical movement.
2. Fill all the wells to the rims (300 µl) with rinsing solution.
3. This rinsing cycle (1 and 2) should be carried out for at least 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 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 at least three rinsing cycles.

- Check the pH and add a few drops of 1 M NaOH to obtain a pH ≥ 8.
- Add 4 ml of tertiary-Butyl-methyl-ether (T-BME) and vortex for 30 seconds.
- Keep the tubes on a table to obtain phase separation.
- Pipette 2 ml of T-BME (upper layer) into a glass tube.
- Evaporate to dryness under a mild stream of nitrogen at a temperature of 50ºC.
- Dissolve the residue in 250 µl sample dilution buffer.
- Use 50 µl portions for the ELISA test.

Sample dilution buffer: Chapter 8, page 12

8. PREPARATION OF REAGENTS

The reagents included in the test-kit are sufficient to carry out at least 96 analyses (including standard analyses). Each standard and sample is analysed in duplicate.

Ready to use standards are prepared in dilution buffer. When an alternative sample matrix is used standards or spikes have to be prepared in the sample matrix from the enclosed 100 ng/ml standard solution. The zero standard should be of the same matrix.

Before starting the test, allow the reagents to come to ambient temperature. Any reagents not used should be put back into storage immediately at +2ºC to +8ºC. Prepare reagents freshly before use.

Keep standard solutions in the dark and store at +2ºC to +8ºC

Microtiter plate
Return unused strips into the zip lock 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 40 ml of diluted rinsing buffer is used (2 ml concentrated rinsing buffer + 38 ml distilled water).

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

Standard solution (100 ng/ml)
To prepare standards in the appropriate matrix or to prepare spikes use the standard solution containing 100 ng salbutamol per ml. Dilute the standard in the sample matrix to make a dilution range of 2.0, 1.0, 0.5, 0.25, 0.125 and 0.062 ng/ml. Also the zero standard should be from the same matrix.

7.2. Faeces, liver, kidney

Remark: Salbutamol is mainly present as a glucuronidated compound. For the determination of salbutamol in combination with extraction procedures, enzymatic hydrolysis is necessary.

- To 1 g of homogenised faeces or tissue (liver or kidney) sample, 5 ml of 0.1 M HCl are added.
- Mix for 1 min. on a vortex and centrifuge for 10 min. at 2000 x g and at 4ºC.
- Transfer 2 ml of the supernatant (approximately 0.4 g of sample) into another tube and add 2 ml of 0.2 M acetate buffer (pH 4.8), check the pH and if necessary adjust the pH to 4.8 ± 0.1 with a few drops of 1 M NaOH.
- Add 20 µl of Helix pomatia juice (Merck art. no. 4114) and incubate overnight at 37ºC or during 2 hours at 55ºC.
- After this incubation, the pH 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 (Merck art. no. 984), mix (vortex) during 30 seconds and centrifuge for 5 min. (2000 x g).
- Pipette 2 ml of the upper layer (isobutanol) into a glass tube and evaporate to dryness under a mild stream of nitrogen at 50ºC. Add 500 µl of sample dilution buffer to the residue and mix (vortex) during 30 seconds (0.4 g of sample/ml buffer). Pipette 2 x 50 µl of this solution in the test-kit.

7.3 Bile and plasma

- To 1 ml of bile or plasma sample 4 ml of 0.1 M HCI is added.
- Mix for 1 min. using a vortex and centrifuge for 10 min. at 2000 x g, at 4ºC.
- Transfer 2 ml of the supernatant (about 0.4 ml of sample) into another tube and add 2 ml of 0.2 M acetate buffer (pH 4.8), check the pH and if necessary adjust the pH to 4.8 ± 0.1 with a few drops of 1 M NaOH.
- Add 20 µl of Helix pomatia juice (Merck art. no. 4114) and incubate overnight at 37ºC or during 2 hours at 55ºC.
- After this incubation, the pH has to be adjusted to 9.8 ± 0.2 by adding 0.25 ml of 1 M Sodium carbonate and a few drops of 1 M NaOH.
- Add 4 ml of isobutanol (Merck art. no. 984), mix (vortex) during 30 seconds and centrifuge for 5 min. at 2000 x g.
- Pipette 2 ml of the upper layer (isobutanol) into a glass tube and evaporate to dryness under a mild stream of nitrogen at 50ºC. Add 500 µl of sample dilution buffer to the residue and mix (vortex) during 30 seconds (0.4 ml sample/ml buffer). Pipette 2 x 50 µl of this solution in the test-kit.

7.4 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.
- The choroid and retina are collected 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 buffer pH 8.0***** containing 1.25 mg pronase E (Sigma***).
- Incubate overnight at 55ºC and centrifuge (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 NaOH (10 M).

- Add 4 ml of isobutanol.
- After vortexing for 30 sec, wait for 5 min (phase separation).
- Subsequently, 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 1 ml of sample dilution buffer.
- If the weight of the choroid/retina sample is 1 g than the dissolved residue contains 0.25 g sample/ml.
- Use 50 µl of the solution in the ELISA test.

7.5 Muscle samples

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 buffer pH 8.0**** containing 1.25 mg pronase E (Sigma***)
- Incubate overnight at 55ºC and centrifuge (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 NaOH (10 M)
- Add 4 ml of isobutanol
- Vortex for 30 sec and wait for 5 min (phase separation)
- 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 50 µl of the solution in the ELISA.

Solid phase extraction method

- Homogenise approximately 10 g of tissue
- Weigh 1 g of the homogenised sample and transfer into a glass tube
- Add 4 ml of 0.1 M Tris buffer** pH 8.0 containing 1.25 mg pronase E (Sigma***)
- Incubate overnight at 55ºC and centrifuge (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 NaOH (10 M)
- Filter the solution through an Aerodisc 0.5 µm PTF1 filter
- For further sample clean-up, use Varian SPEC MP1 columns no. A5321130
- Activate the columns by washing successively with 250 µl of 100% methanol and 250 µl of 0.1 M acetic acid
- Transfer 1 ml of the filtrate onto the columns and draw it slowly through the columns, flow 1 ml/min (do not dry the columns!!)
- Wash the columns with 500 µl of a solution of 100% methanol/0.1 M Acetic acid (1:1; V:V)
- Elute the β-agonists with 3 ml of 2% ammonium hydroxide in ethyl acetate****
- Evaporate the eluent to dryness at 50ºC under a mild stream of nitrogen
- Dissolve the residue in 500 µl of sample dilution buffer
- Use 50 µl of the solution in the ELISA.

** Tris buffer pH 8.0 dissolve 24.2 g Tris and 14.7 g $CaCl_2$ in 1 litre H_2O, adjust pH.

*** Pronase E from *Streptomyces griseus* (9036-06-0; article number P5147 of Sigma.

**** After preparation this solution appears turbid. Prepare the solution at least 12 hours before use and leave at room temperature. The solution will become clear.

7.6 Feed samples

- Approximately 50 – 100 g of sample is ground and pulverised into a fine powder.
- To 5 g of the ground feed sample, 25 ml of 100 % methanol and 25 ml of 0.2 M phosphoric acid are added.
- Shake for 30 minutes at room temperature and centrifuge for 15 min. at 2000 x g and at 4ºC.
- Pipette 0.25 ml of the supernatant and add 1 ml of sample dilution buffer, check the pH (7.4 ± 0.2) and adjust if necessary by a few drops of 1 M NaOH.
- Mix (vortex) for 30 seconds and centrifuge at 2000 x g for 10 min.
- Use 50 µl of the supernatant in the ELISA (equals 0.02 g of feed sample per ml).

7.7 Hair samples

- An amount of 0.1 g of pre-washed hair is weighed into a test-tube (9 x 1.4 cm) and 2.5 ml of 5 M NaOH is added.
- The mixture is heated for 10 min. at 95ºC in water-bath.
- Cool to room temperature and add 5 ml of tertiair butyl methyl ether (T-BME).
- Mix the content in the tubes for 30 min. (head over head).
- Centrifuge the mixtures for 5 min. at 2000 x g.
- Transfer 2.5 ml of upper layer (T-BME), containing the extracted β-agonists, into another tube and evaporate at 50ºC under a mild stream of nitrogen.
- Dissolve the residue in 500 µl of sample dilution buffer and pipette 50 µl into the wells of the microtiter plate.
- This method is only suitable for the detection of Clenbuterol, Bromobuterol, Mapenterol and Mabuterol [8].

7.8 Skimmed milk powder and Milk samples

- To 100 g of skimmed milk powder an amount of 600 ml distilled water is added.
- The solubilised skimmed milk samples and milk samples are treated as follows:
- Defat approximately 5 ml of milk sample.
- Store the milk samples at 4ºC followed by centrifugation for 10 min at 4ºC and at 2000 x g.
- Remove the upper fat layer.
- Transfer 1 ml of defatted milk into a glass tube.
- Add 1 ml of 0.1 M di-Sodium-tetraborate, pH 8.

Sample dilution buffer: Chapter 8, page 12