RACTOPAMINE ELISA

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

EUROPROXIMA RACTOPAMINE ELISA

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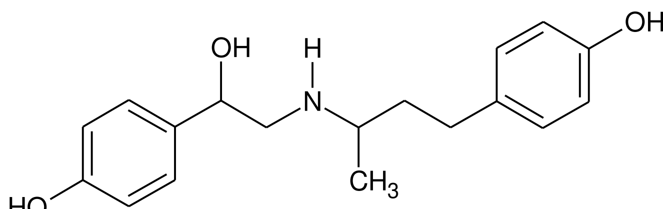
BRIEF INFORMATION

The ractopamine ELISA is a competitive enzyme immunoassay for the screening of various samples. The test is based on antibodies directed against ractopamine. The ELISA kit contains a 96 well microtiter plate as well as all essential reagents including ready-to-use standards to perform the test.

1. INTRODUCTION



Chemical structure of ractopamin

Ractopamine is a β-adrenergic agonist which acts as a nutrient repartitioning agent in livestock by diverting nutrients from fat deposition to the production of muscle tissues. Ractopamine is a growth promoter, its use is forbidden in EU countries. The US Food and Drug Administration (FDA) approved ractopamine as a swine feed supplement. As a result all meat exported to EU must undergo a primary screening by a rapid method such as an immunoassay.
EuroProxima B.V. has developed an enzyme immunoassay for screening of biological samples on the presence of ractopamine.

2. PRINCIPLE OF THE RACTOPAMINE-ELISA

The microtiter plate based ractopamine ELISA consists of one precoated plate (12 strips, 8 wells each). Horseradish peroxidase (-HRP) labeled ractopamine and standard solution or sample are added to the wells. Free ractopamine from the samples or standards and ractopamine-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 ractopamine-HRP conjugate is visualized by the addition of a substrate/chromogen solution (H_2O_2/TMB). Bound ractopamine-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 ractopamine concentration in the sample.

3. SPECIFICITY AND SENSITIVITY

The ractopamine ELISA utilizes antibodies raised in rabbit against protein conjugated ractopamine.

Cross- reactivity:	Ractopamine	100%
	Clenbuterol	< 0.1%
	Fenoterol	< 0.1%
	Ritodrine	< 0.1%
	Salbutamol	< 0.1%
	Salmeterol	< 0.1%
	Isoxsuprine	< 0.1%

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	8.1	0.04
Tissue	8.2	0.1
Liver	8.2	0.4
Feed	8.3	2.0
Serum	8.4	0.4
Urine	8.5	1.0

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:

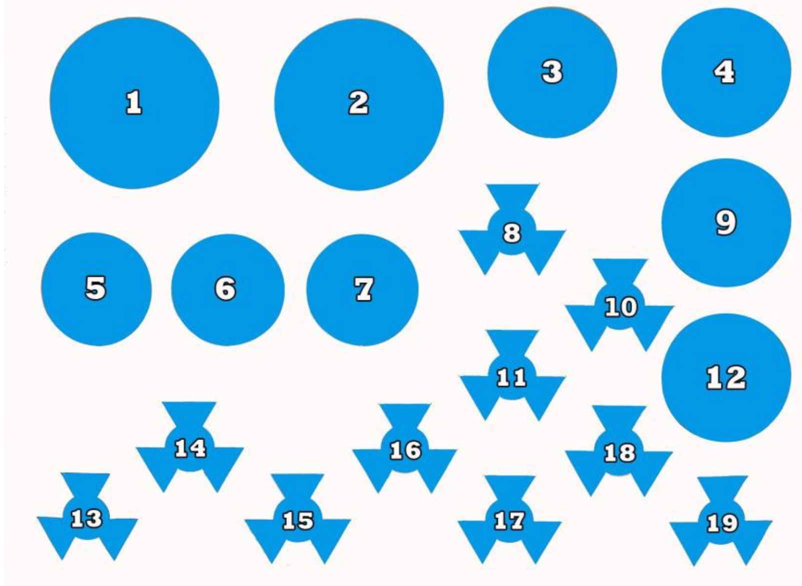
- A blue colouring of the chromogen solution before transferring it into the wells.
- A weak or no colour reaction in the zero standard wells (E450nm < 0.8).

5. KIT CONTENTS

Manual

One sealed (96-wells) microtiter plate (12 strips, 8 wells each), coated with antibody. Plate is ready-to-use.
One bag with 10 gram skimmed milk powder.

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. **Conjugate** (lyophilised, blue cap)
6. **Conjugate** (lyophilised, blue cap)
7. Not in use
8. **Standard solution** (1 ml, Ready-to-use) 100 ng/ml
9. Not in use
10. Not in use
11. Not in use
12. Not in use
13. **Zero standard solution** (2 ml, Ready-to-use)
14. **Standard solution 1** (1 ml, Ready-to-use) **0.063 ng/ml**
15. **Standard solution 2** (1 ml, Ready-to-use) **0.125 ng/ml**
16. **Standard solution 3** (1 ml, Ready-to-use) **0.25 ng/ml**
17. **Standard solution 4** (1 ml, Ready-to-use) **0.5 ng/ml**
18. **Standard solution 5** (1 ml, Ready-to-use) **1 ng/ml**
19. **Standard solution 6** (1 ml, Ready-to-use) **2 ng/ml**

6. EQUIPMENT AND MATERIALS REQUIRED BUT NOT PROVIDED

- 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
- Acetonitril (CH_3CN)

7.PRECAUTIONS

- **T**his 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.

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8. SAMPLE PREPARATIONS

8.1 Milk samples

- Pipette 2 ml milk into 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 2000 x g at 20°C - 25°C
  Alternative: allow a supernatant to develop
- Pipette 2 ml from the supernatant into a glass tube
- Evaporate to dryness under a mild stream of nitrogen or compressed air at 50°C
- Dissolve the residue in 250 µl of dilution buffer, vortex vigorously
- Use 25 µl of this solution in the ELISA test

8.2Tissue and Liver

- Remove fat and homogenize sample to a fine mass, use stomacher of mixer
- Transfer 1 g of the homogenized sample into a centrifugal screw cap vial, add 4 ml of acetonitrile and mix well (vortex)
- Shake for 30 minutes head over head (rotor)
- Centrifuge 10 minutes at 2000 x g at 20°C - 25°C
- Transfer 1 ml of supernatant into a new centrifugal vial and evaporate to dryness under a mild stream of nitrogen or compressed air at 50ºC
- The residue is dissolved in 500 µl dilution buffer, vortex rigorously
- An aliquot of 50 µl is diluted with 50 µl dilution buffer, vortex
- Use 25 µl of this solution in the ELISA test

8.3 Feed samples

- Grind a representative amount of feed sample
- Mix 2 g of feed sample with 8 ml of acetonitrile
- Vortex for 1 minute
- Mix head-over-head during 10 minutes at 20°C - 25°C
- Centrifuge 10 minutes at 2000 x g at 20°C - 25°C
- Pipette 0.5 ml of the supernatant into a glass tube
- Evaporate to dryness under a mild stream of nitrogen or compressed air at 50°C
- Dissolve the residue in 500 µl of dilution buffer, vortex vigorously
- Mix 100 µl of the dissolved sample with 400 µl of sample dilution buffer (see chapter 9) for feed and urine
- Use 25 µl of this solution in the ELISA test

8.4 Serum samples

- Pipette 1 ml serum in a clean tube
- Add 4 ml acetonitrile and mix head over head for 10 minutes
- Centrifuge 10 minutes at 2000 x g at 20°C - 25°C
- Pipette 1 ml from the clear upper layer into a glass tube
- The acetonitrile is evaporated to dryness under a mild stream of nitrogen or compressed air at 50°C
- The residue is dissolved in 400 µl dilution buffer, vortex rigorously
- Use 25 µl of this solution in the ELISA test

8.5 Urine samples

- When the urine is turbid centrifuge 5 minutes at 2000 x g at 20°C - 25°C
- Mix 100 µl of urine sample with 400 µl of sample dilution buffer (see chapter 9) for feed and urine.
- Use 25 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 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 sample dilution buffer is 4 times concentrated. Before dilution (20 ml buffer + 60 ml distilled water) the concentrated buffer should be at 20°C to 25°C and thoroughly mixed. Concentrated buffer can show precipitates of the contents. Mix well before dilution with distilled water.

Sample dilution buffer

Add 1 g of skimmed milk powder per 8 ml of dilution buffer.

Conjugate solution

Reconstitute the vial of lyophilised conjugate (ractopamine-HRP) with 5 ml of dilution buffer), mix thoroughly and keep in the dark until use.

Rinsing buffer
The rinsing buffer is delivered 20x concentrated. Prepare dilutions freshly before use. For each 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 ractopamine 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 20°C to 25°C when used (keep in the dark) and mix the content before pipetting into the wells.

10. 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 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 (ractopamine-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.

5. Incubate the plate for 30 minutes in the dark at 20°C to 25°C.

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

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

8. Incubate 30 minutes in the dark at 20°C to 25°C.

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

10. Read the absorbance values immediately at 450 nm.

11. INTERPRETATION OF RESULTS

Subtract the mean optical density (O.D.) value of the blank wells H1 and H2 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.

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

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 absorbtion 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.

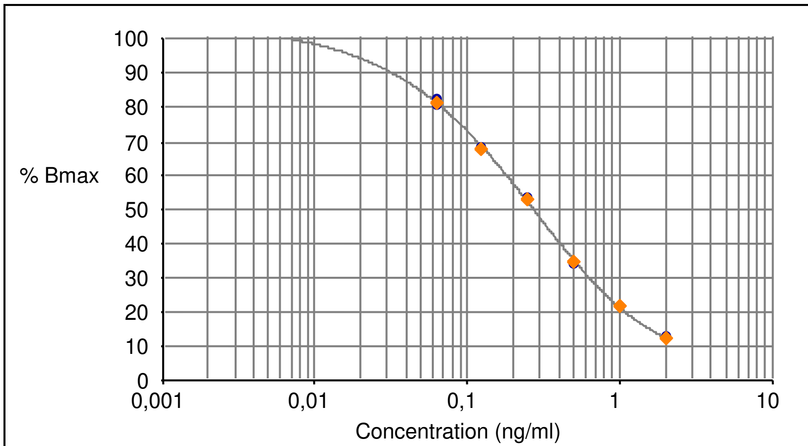

Figure 1: Example of a calibration curve

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

<u>8.1 Milk</u>
The ractopamine equivalents read from the standard curve has to be multiplied by a factor 0.625.

<u>8.2 Tissue and liver</u>
The ractopamine equivalents read from the standard curve has to be multiplied by a factor 5.

<u>8.3 Feed</u>
The ractopamine equivalents read from the standard curve has to be multiplied by a factor 25.

<u>8.4 Serum</u>
The ractopamine equivalents read from the standard curve has to be multiplied by a factor 2.

<u>8.5 Urine</u>
The ractopamine equivalents read from the standard curve has to be multiplied by a factor 5.

12. LITERATURE

Not applicable.

13. ORDERING INFORMATION

For ordering the ractopamine ELISA kit please use cat. code 5061RACT

14. REVISION HISTORY

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

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