CORTICOSTEROID ELISA

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

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TABLE OF CONTENTS

PAGE:

Brief Information 2
1. Introduction..2
2. Principle of the corticosteroid ELISA 3
3. Specificity and Sensitivity...4
4. Handling and Storage 5
5. Kit contents..6
6. Equipment and materials required but not provided........7
7. Precautions 7
8. Sample preparation..8
9. Preparations of reagents...10
10. Assay Procedure 10
11. Interpretation of results 12
12. Literature 13
13. Ordering information 14
14. Last mutations 14

13. ORDERING INFORMATION

For ordering the Corticosteroid ELISA kit, please use cat. code 5081COR.

14. LAST MUTATIONS

Adapted standard line.
Pipette schedule of the standard curve is adapted.

EuroProxima B.V. TEL: + 31 26 3630364
Beijerinckweg 18 FAX: + 31 26 3645111
NL 6827 BN Arnhem Web-site:http://www.europroxima.com
The Netherlands E-mail: info@europroxima.com

8.2. Urine samples

8.2.1 Using the direct method, the equivalents have to be multiplied by a factor 50 (0.02 ml of urine/ml).

8.2.2 Using the extraction procedure the equivalents can directly be read from the standard curve (factor 1).

8.3 Tissue samples:

8.3.1 Extraction procedure I
Using procedure I the results read from the standard curve have to be multiplied by a factor 2.

N.B. Extraction procedure I
Using procedure I and a fat extraction the results read from the standard curve have to be multiplied by a factor 4.

8.3.2 Extraction procedure II
Using procedure II the results can directly be read from the standard curve (factor 1).

8.4 Feed samples:

8.4.1 Extraction procedure I
Using Procedure I for sample preparation, the dexamethasone equivalents can directly be read from the standard curve (factor 1).

8.4.2 Extraction procedure II
Using Procedure II for sample preparation, these dexamethasone equivalents have to be multiplied by a factor 100 (0.01 g feed/ml).

Remark
"Positive" samples found with this ELISA have to be confirmed with analytical methods such as LC-MS or GC-MS.

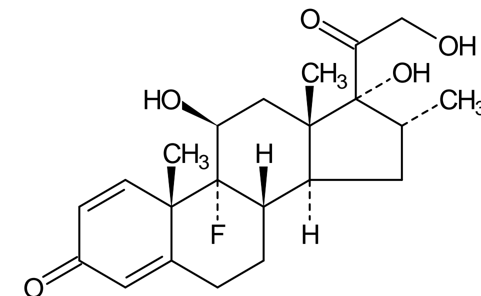
12. LITERATURE

1. Council of the European Communities, Council Directive 86/469/EC of 16 September 1986.
2. Brunn. H and Georgii, S. Identification and quantification of dexamethasone and related xenobiotic corticosteroids in cattle urine with ELISA and HPLC/ELISA. Archiv für Lebensmittel Hygiene 45 (4), 1994, 96.
3. P. Stouten, W. Haasnoot, G. Cazemier, P. Berende and H. Keukens; Immunochemical detection of corticosteroids in milk, liver, kidney and muscle. Proceedings Euro-Residue III 1996 ed. N. Haagsma, A. Ruiter, p. 902.
4. Commission Regulation (EU) No. 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin. Off. J. European Union L15, 1-72.

BRIEF INFORMATION

The Corticosteroid ELISA is a competitive enzyme immunoassay for measurement of the concentration of corticosteroids such as dexamethasone, betamethasone, flumethasone, triamcinolone and prednisolone. With this ELISA-kit 96 analyses can be performed. Samples and standards are measured in duplicate which means that a total 40 samples can be analysed.
The ELISA kit contains all reagents to perform the assay. Reagents for sample preparation are not included in the kit.

1. INTRODUCTION



Chemical structure of dexamethasone

Synthetic corticosteroids, are widely used in live-stock breeding. After application of such corticosteroids a delay time of 48 hours for milk and 72 hours for meat is prescribed. Corticosteroids are found in cattle feed and are suspected to be used as growth-promoters. In all European Community countries however, the use of growth promoters has been banned since 1986 [1].
With this ELISA kit milk, urine, tissue and feed samples can be screened in a short time for the presence of corticosteroids such as dexamethasone, betamethasone, flumethasone and other cross-reacting corticosteroids [2,3].
This test makes no distinction between the different corticosteroids and can therefore only be used as a screening method. For the confirmation of the presence and the identification of the specific steroid, more specific analytical methods (LC-MS or GC-MS) are recommended.

Within the EU Maximum Residue Limits (MRLs) for dexamethasone, betamethasone, prednisolone and methylprednisolone are established in milk, muscle (fat), kidney and liver [4].
These MRLs can be summarised as follows:

Corticosteroid	Muscle	Liver	Kidney	Milk
Dexamethasone	0.75 µg/kg	2 µg/kg	0.75 µg/kg	0.3 µg/kg
Betamethasone	0.75 µg/kg	2 µg/kg	0.75 µg/kg	0.3 µg/kg
Prednisolone	4 µg/kg	10 µg/kg	10 µg/kg	6 µg/kg
Methylprednisolone	10 µg/kg (including fat)	10 µg/kg	10 µg/kg	Not for use in animals from which milk is produced for human consumption

2. PRINCIPLE OF THE CORTICOSTEROID ELISA

The microtiter plate based corticosteroid ELISA consists of one precoated plate (12 strips, 8 wells each). Antibody, Horseradish peroxidase (-HRP) labeled dexamethasone (enzyme conjugate) and dexamethasone standard solution or samples are added to the wells. The specific antibodies are bound by the immobilised anti-rabbit antibodies and at the same time dexamethasone (in the standard solution or in the samples) and enzyme labelled dexamethasone compete for the specific antibody binding sites (competitive enzyme immunoassay).
After an incubation time of one hour, the non-bound reagents are removed in a washing step. The amount of bound dexamethasone-HRP conjugate is visualized by the addition of a substrate/chromogen solution (H_2O_2/tetramethylbenzidine, TMB). Bound dexamethasone-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 corticosteroid concentration in the sample.

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.

O.D. standard (or sample)
--------------------------------------x 100% = % maximal absorbance
O.D. zero standard/ Bmax

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 value of absorption (logit) calculation of the standards are plotted on Y-axis versus the analyte equivalent concentration on a logarithmic X-axis.

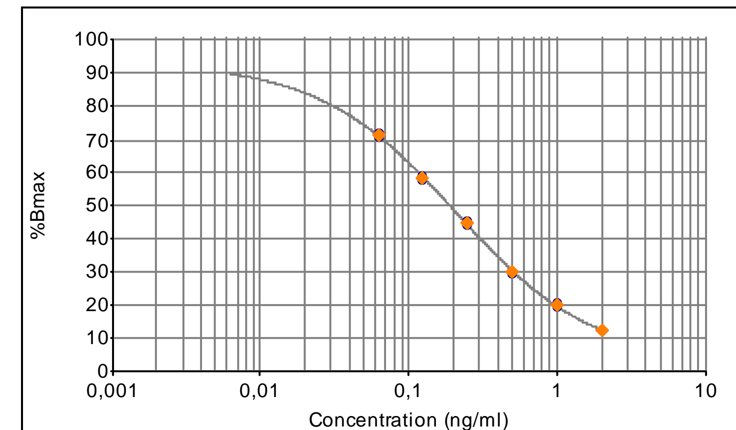


Figure 1: Example of a calibration curve

8.1 Milk samples:
The equivalents (ng/g of milk) corresponding to the % maximal OD of each sample can directly be read from the calibration curve (no dilution applied).

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 (sample preparation) and prepare reagents according to chapter 9 (preparation of reagents).

2. Pipette 100 µl of zero standard in duplicate (wells H1, H2, blank).
   Pipette 50 µl of zero standard in duplicate (wells A1, A2).
   Pipette 50 µl of each standard solution in duplicate (wells B1, B2 to G1, G2 i.e. 0.0625, 0.125, 0.25, 0.5, 1.0 and 2.0 ng/ml).

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

4. Pipette 25 µl of conjugate (DEX-HRP) to all wells, except wells H1 and H2.

5. Pipette 25 µl of antibody solution to all wells, except wells H1 and H2.

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

7. Incubate for 1 hour in the dark at room temperature (20ºC to 25ºC).

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

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

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

11. Pipette 100 µl of stop solution into each well.

12. Read the absorbance values immediately at 450 nm.

3. SPECIFICITY AND SENSITIVITY

The corticosteroid ELISA utilises antibodies raised in rabbits against protein conjugated dexamethasone. The reactivity pattern of the antibody is:
Cross-reactivity:

Compound	Cross-reactivity
Flumethasone	125%
Dexamethasone	100%
Betamethasone	93%
Triamcinolone	33%
Prednisolone	21%
Dianabol	7.5%
Methylprednisolone	2.8%
20β-hydroxyprogesterone	1.8%
Cortisol	1.8%
Progesterone	0.8%
17β-Testosterone	0.7%
Corticosterone	0.6%
17β-19-Nortestosterone	0.1%
Medroxy-Progesterone-Acetate	< 0.1%
Ethisterone	< 0.1%

REMARK: Although not tested, the antibodies will probably show high cross-reactivities with dexamethasone-21 and betamethasone-21 derivatives.

For extraction procedures the LOD is calculated reflecting the respective concentration steps. Cut-off values need critical consideration.

The limit of detection (LOD) in ppb is calculated as: Xn+3SD and is determined under optimal conditions.

	8.1 Milk Direct	8.3.1 Muscle Procedure I	8.3.2 Liver Procedure II	8.2.1 Urine Direct	8.2.2 Urine Extraction	8.4.1 Feed Procedure I
Dexamethasone	0.2	0.2	1	12	3	0.6
Betamethasone	0.2	0.2	1	12	3	0.6
Flumethasone	0.2	0.2	1	12	3	0.6
Triamcinolone	0.6	0.6	3	36	9	1.8
Prednisolone	1.0	1.0	5	60	15	3.0

4. HANDLING AND STORAGE

- Store the kit 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 (see kit label) has passed, quality claims are not accepted.
- Before opening the sealed plate, the plate should be at ambient temperature in order to avoid condensation in the ELISA.
- Dilute the kit components immediately before use, but after the components are at ambient temperature.
- After the lyophilised antibody and conjugate have been reconstituted, the antibody and conjugate solutions are stable for maximally one week when stored in a refrigerator at +2ºC to + 8ºC. 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 chromogen solution 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.

Degeneration of the reagents may have occurred when the following phenomena are observed:
- A blue colouring of the substrate solution before transferring it into the wells,
- A weak or absent colour reaction of the zero standard (Bmax; E450 nm < 0.8).

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 +2°C to +8ºC. Prepare reagents fresh before use.

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

Rinsing buffer
The rinsing buffer is delivered 20x concentrated. Prepare dilutions freshly before use. For each strip 40 ml of diluted rinsing buffer is used (2 ml concentrated rinsing buffer + 38 ml distilled water).

Substrate/chromogen solution (12 ml)
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.

Conjugate
Reconstitute the vial of lyophilised conjugate (DEX-HRP) with 4 ml of 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: freeze aliquots at -20ºC.

Antibody
Reconstitute the vial of lyophilised antibodies with 4 ml of 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: freeze aliquots at -20ºC.

10. ASSAY PROCEDURE

Rinsing protocol

In ELISAs, 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.

8.3.2. Extraction procedure II

- Produce approximately 10 to 50 g of minced tissue sample
- To 2 g of the minced tissue sample, 10 ml of a mixture of acetonitrile/ water (7:3 v/v) is added
- Mix (vortex) for 1 minute
- Shake the samples (head over head) for approximately 30 minutes
- Centrifuge for 10 minutes at 2000 x g at 4ºC
- Pipette 2.5 ml of the supernatant into a glass tube
- Defat by addition of 4 ml of hexane and 1 ml of dichloromethane
- Mix (vortex) for 1 minute and allow the 3 phases to separate
- Pipette 1 ml from the middle phase (corresponding to 0.2 g of sample) into a clean tube
- Evaporate to dryness under a mild stream of nitrogen at a temperature of 50ºC
- Dissolve the residue in 0.2 ml of Phosphate Buffer/Tween*
- Use 50 µl in the ELISA

8.4 Feed samples

8.4.1 Extraction procedure I

- Grind 10 to 100 g of feed sample
- Homogenize 1 g of the grinded feed sample with 4 ml ethyl acetate
- Shake for 30 minutes, e.g. head over head
- Centrifuge for 10 minutes at 2000 x g at 4ºC
- Pipette 1 ml of the supernatant into a clean glass tube and evaporate to dryness under a mild stream of nitrogen at a temperature of 50ºC
- Dissolve the residue in 0.25 ml of Phosphate Buffer/Tween*
- Use 50 µl in the ELISA

8.4.2 Extraction procedure II

- Grind 10 to 100 g of feed sample
- Homogenize 1 g of the grinded feed sample with 3 ml of distilled water
- Add 7 ml of acetonitrile
- Shake for 30 minutes, e.g. head over head
- Wait for 5 minutes to obtain a separation between the solid part and the liquid part
- Pipette 2.5 ml of the clear upper part into a 10 ml tube
- Add 1 ml of dichloromethane and 4 ml of hexane
- Shake for 1 minute and allow the 3 phases to separate
- Take 100 µl of the middle phase and evaporate to dryness
- Add 1 ml of Phosphate Buffer/Tween* to the dry residue
- Mix and use 50 µl of this solution in the ELISA (0.01 g feed/ml).

* Phosphate Buffer/Tween

Dissolve in 1000 ml water:

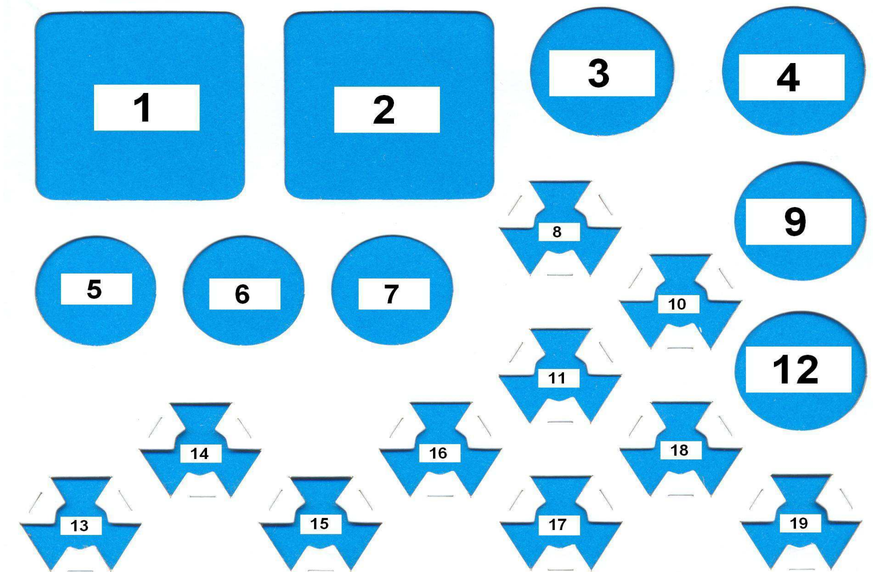
Na_2HPO_4	1.15 g
KH_2PO_4	0.2 g
KCl	0.2 g
NaCl	30 g
Tween 80	0.5 ml
BSA (Sigma A 7030)	10 g

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, 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 Solution** (lyophilized, blue cap)
6. **Antibody solution** (lyophilized, yellow cap)
7. not in use
8. not in use
9. not in use
10. not in use
11. not in use
12. not in use
13. **Zero standard** (2ml, ready-to-use)
14. **Standard solution 1** (1ml, ready-to-use) **0.0625 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

- 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
- Helix pomatia juice (Merck 1.04114.0002)
- Ethyl acetate
- Hexane
- Dichloromethane
- Acetonitrile
- Methanol 100%
- 4 ml glass tubes
- 15 ml tubes with screw cap (Greiner, polypropylene)
- 0.2 M sodium acetate buffer pH 4.8

7. PRECAUTIONS

- Dexamethasone is a toxic compound. Avoid contact with mouth and skin. Be aware that dexamethasone is not inhaled.
- The stop reagent contains 0.5 M sulfuric 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; take care when handling the substrate.
- 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, which crystallises 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 Defatted milk.

Milk samples are analysed directly after defatting (centrifuge for 10 minutes at 2000 x g at 4ºC). 50 µl of the defatted milk is pipetted into the microtiter plate.

8.2 Urine.

8.2.1 Procedure I / Direct method.

To 100 µl of urine, 4.9 ml of buffer (Phosphate Buffer/Tween*) is added.

8.2.2 Procedure II / Extraction procedure

- Pipette 1 ml of urine in a clean tube
- Add 1 ml of 0.2 M sodium acetate buffer pH 4.8
- Check the pH and, if necessary, adjust the pH to 4.8 with a few drops of 1 M acetic acid
- Add 10 µl of Helix pomatia juice (10 times diluted in distilled water)
- Incubate overnight at 37ºC or during 2 hours at 55ºC
- Cool to room temperature
- Adjust the pH to 7.4 ± 0.4 by addition of 100 µl of 0.5 M NaOH
- Add 4 ml of ethyl acetate
- Mix for 15 minutes, e.g. head over head, at room temperature
- Centrifuge for 10 minutes at 2000 x g at 4ºC
- Pipette 2 ml of the supernatant into a clean glass tube and evaporate to dryness under a mild stream of nitrogen at a temperature of 50ºC
- Dissolve the residue in 0.5 ml of Phosphate Buffer/Tween*
- Use 50 µl in the ELISA

8.3 Tissue samples (muscle, liver)

8.3.1. Extraction procedure I

- Produce approximately 10 to 50 g of minced tissue sample
- To 1 g of the minced tissue sample, 3 ml of distilled water is added
- Mix (vortex) for 1 minute
- Add 4 ml of ethyl acetate
- Shake the samples (head over head) for approximately 30 minutes
- Centrifuge for 10 minutes at 2000 x g at 4ºC
- Pipette 1 ml of the supernatant into a glass tube
- Evaporate to dryness under a mild stream of nitrogen at a temperature of 50ºC
- Dissolve the residue in 0.5 ml of Phosphate Buffer/Tween*
- Use 50 µl in the ELISA

N.B. When after evaporation a fatty residue is obtained, dissolve the residue in 1 ml of hexane, add 1 ml of Phosphate Buffer/Tween*, vortex for 1 minute and centrifuge (10 minutes at 2000 x g). Use 50 µl of the phase underneath in the ELISA.