DIETHYLSTILBESTROL ELISA

5081DES[4]02.20

A competitive enzyme immunoassay for screening and quantitative analysis of diethylstilbestrol (DES) in various matrices

EUROPROXIMA DIETHYLSTILBESTROL ELISA

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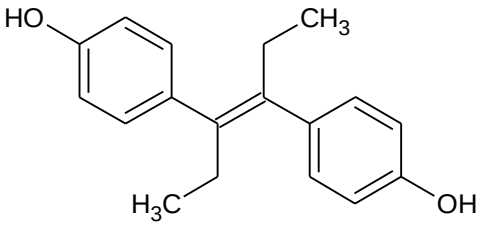
BRIEF INFORMATION

The DES ELISA is a competitive enzyme immunoassay for measurement of the concentration of diethylstilbestrol in tissue and urine. 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 analysed with one kit.
The ELISA kit contains all the reagents needed to perform the assay. Reagents for sample preparation are not included in the kit.

1. INTRODUCTION



Chemical structure of diethylstilbestrol (DES)

Diethylstilbestrol (DES), together with dienestrol and hexestrol, belongs to the group of stilbenes. DES is an oestrogenic compound which affects weight gain and feed conversion efficiency if administered to animals used for meat production. The increased weight gain and improved feed conversion efficiency has been observed in cattle, chicken and lambs. However, in the EC, the use of substances having a hormonal action on growth promotion in farm animals was banned in 1981 due to the harmful residues these substances leave in meat.
The DES ELISA can be used to screen tissues and urine for the presence of this illegal compound.

2. PRINCIPLE OF THE DES ELISA

The microtiter plate based DES ELISA consists of one plate (12 strips, 8 wells each) precoated with antibodies. Standard solution or sample and horseradish peroxidase (-HRP) labeled DES are added to the wells. Free DES from the samples or standards and DES-HRP conjugate 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 DES-HRP conjugate is visualized by the addition of a substrate/chromogen solution (H_2O_2, TMB). Bound DES-HRP conjugate transforms the colourless 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. The optical density is inversely proportional to the DES concentration in the sample.

3. SPECIFICITY AND SENSITIVITY

The DES ELISA utilizes antibodies raised in rabbits against protein conjugated diethylstilbestrol. The reactivity pattern of the antibody is:

Cross-reactivity:	Diethylstilbestrol	100%
	Diethylstilbestrol-glucuronide	68%
	Hexestrol	22%
	Dienestrol	< 1%
	17 ß-Estradiol	< 0.01%

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) and the detection capability (CCβ) are determined under optimal conditions. Cut-off criteria need critical consideration.

Matrix	Procedure	LOD [ppb]	CCβ [ppb]
Tissue	8.1	0.18	0.25
Urine	8.2	0.16	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:

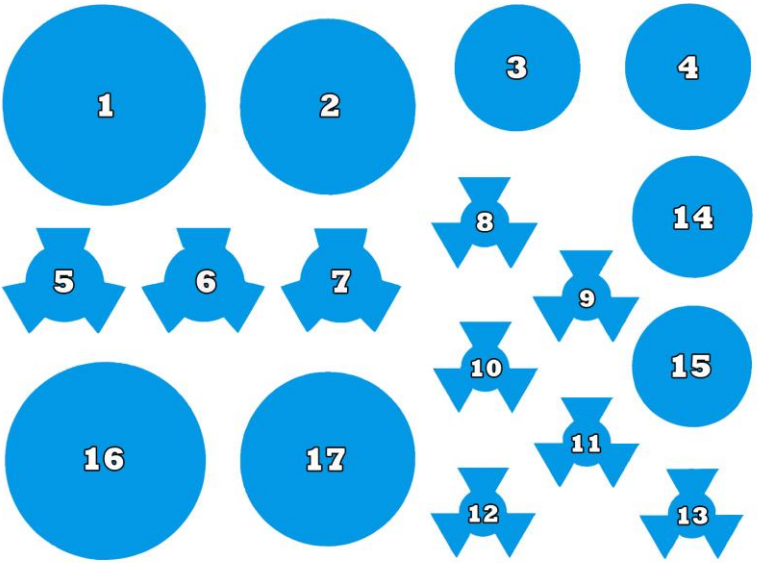
- A blue colouring of the chromogen solution before transferring it into the wells.
- A weak or no colour reaction in the zero standard wells (E450nm < 0.8).

5. KIT CONTENTS

Manual

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

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



1. **Dilution buffer** (40 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** (100 µl; 100x concentrated
6. not in use
7. **Zero standard solution** (2ml, Ready-to-use)
8. **Standard solution 1** (1ml, Ready-to-use) **0.0313 ng/ml**
9. **Standard solution 2** (1ml, Ready-to-use) **0.0625 ng/ml**
10. **Standard solution 3** (1ml, Ready-to-use) **0.125 ng/ml**
11. **Standard solution 4** (1ml, Ready-to-use) **0.25 ng/ml**
12. **Standard solution 5** (1ml, Ready-to-use) **0.5 ng/ml**
13. **Standard solution 6** (1ml, Ready-to-use) **1.0 ng/ml**
14. **Conjugate diluent** (15 ml, Ready-to-use)
15. not in use
16. not in use
17. not in use

6. EQUIPMENT AND MATERIALS REQUIRED BUT NOT PROVIDED

- Polypropylene tubes with screw cap (Greiner 188271)
- Scales and weighing vessels
- Glass tubes
- Gloves
- Fume hood
- Homogeniser
- Vortex, mixer
- Centrifuge (4000 x g)
- Automated microtiter plate washer or 8-channel micropipette 100 – 300 µl
- Microtiter plate shaker
- Microtiter plate reader with 450 nm filter
- Micropipettes 0.5 – 10 µl
- Micropipettes, 100 – 1000 µl
- Multipipette with 2.5 ml combitips
- Glucuronidase / arylsulfatase of Helix pomatia (Merck 1.04114.0002)
- Methanol anhydrous
- Ethyl acetate

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 PREPARATIONS

8.1 Meat/Tissue samples
- Weigh 1 gram of homogenized meat into a polypropylene tube (use only muscle tissue without fat and connective tissue)
- Add 5 ml of ethyl acetate and vortex at maximum speed for 2 minutes
- Centrifuge for 5 minutes at 4000 x g
- Collect 2.5 ml of the upper layer (ethyl acetate) in a new glass tube
- Evaporate under a mild stream of nitrogen at 70°C
- Dissolve the residue in 400 µl of methanol (anhydrous) and vortex for 10 seconds
- Add 600 µl of dilution buffer and vortex at maximum speed for 1 minute
- Centrifuge for 5 minutes at 4000 x g
- Dilute 50 µl of extract with 150 µl of *Sample Dilution Buffer, vortex
- Use 25 µl of the diluted sample per well.

8.2 Urine samples
- Dilute 1 ml urine with 2.5 ml **50 mM sodium acetate buffer, pH 4.6
- Add 20 µl of ten-fold diluted (with distilled water) glucuronidase/arylsulfatase of Helix pomatia
- For hydrolization incubate the solution for 3 hours at 37°C (alternatively over-night at room temperature)
- The hydrolyzed product is further purified as described below

Purification with SPE C18 column:

- **Flow rate: 1 drop per second**
- Rinse the column with 3 ml of methanol (anhydrous)
- Equilibrate the column with 2 ml of ***methanol/20 Mm Tris-HCI, (20/80 v/v), pH 8.5
- Apply sample
- Rinse the column with 2 ml of ***methanol/20 Mm Tris-HCI, (20/80 v/v), pH 8.5
- Rinse the column with 3 ml of methanol/distilled water (40/60 v/v)
- Remove solvent residues by applying positive pressure or vacuum and dry column for 2 minutes by purging it with air or nitrogen
- Elute the analytes with 1 ml of methanol/distilled water (80/20 v/v) into a new vial **(flow rate 15 drops per minute)**
- Collect the eluate residues into the same vial by applying positive pressure or vacuum
- Dilute the eluate 1:1 (100 µl + 100 µl) with dilution buffer supplied in the kit
- Vortex
- Dilute further 1:1 with *Sample Dilution Buffer (100 µl + 100 µl)
- Vortex
- Use 25 µl per well

* Sample Dilution Buffer: see chapter 9

** 50 mM sodium acetate buffer, pH 4.8 : dissolve 0.41 g of sodium acetate in 100 ml of distilled water, adjust the pH to 4.8 with 20% acetic acid

*** Methanol/Tris-HCl (20/80 v/v): dissolve 2.42 g of tris-(hydroxymethyl)-aminomethane in approx. 700 ml of distilled water + 200 ml of methanol (anhydrous), adjust the pH to 8.5 with 5 M HCl and fill up to 1000 ml with distilled water

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 freshly before use.

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

Dilution buffer
The dilution buffer (ready to use) is used to dilute the urine samples after column purification, to dilute the tissue residue dissolved in methanol and to prepare the Sample Dilution Buffer.

*Sample dilution buffer
40% methanol in dilution buffer.
Add 4 ml of anhydrous methanol to 6 ml of dilution buffer.

Conjugate solution (100 µl)
The conjugate is delivered 100x concentrated. Spin down the conjugate in the vial by a short centrifugation step (1 minute, 1000 x g). Add 13 µl of the concentrated conjugate solution to 1287 µl of the conjugate diluent. Per 2 x 8 wells 1300 µl is required. Store the unused concentrated conjugate at 2°C to 8°C.

Rinsing buffer (30 ml)
The rinsing buffer is 20 times concentrated. Prepare fresh dilution before use. Per strip 40 ml of diluted rinsing buffer is used (2 ml concentrated rinsing buffer + 38 ml distilled water).

Substrate solution (12 ml)
The substrate solution (ready to use) precipitates at 4°C. Bring the vial to room temperature (20°C to 25°C, kept in the dark) and mix it well before pipetting into the wells.

10. ASSAY PROCEDURE

Rinsing protocol

Unbound components have to be removed efficiently between each incubation step in ELISAs. This is achieved by appropriate rinsing. Each rinsing procedure must be carried out with care to guarantee good inter- and intra-assay results.
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 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. Do not allow the wells dry out before the next reagent is dispensed

Rinsing with automatic microtiter plate washing equipment

When using automatic plate wash equipment, make sure that all wells can be aspirated completely and 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 section 8 (Sample preparation) and prepare reagents according to section 9 (Preparation of reagents).

2. Pipette 100 µl of zero standard in duplicate (blank; well H1, H2).
   Pipette 25 µl of zero standard in duplicate (zero standard; well A1, A2).

3. Pipette 25 µl of each standard dilution in duplicate (B1,2 to G1,2)

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

5. Add 75 µl diluted conjugate (diethylstilbestrol HRP) to all wells, except wells H1 and H2.

6. Seal the microtiter plate and shake the plate a few seconds.

7. Incubate for 1 hour in the dark at room temperature (20°C - 25°C).

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

9. Pipette 100 µl substrate solution into each well.

10. Incubate 30 min. at room temperature (20°C - 25°C).

11. Add 100 µl stop solution to each well.

12. 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.

$$\frac{\text{OD standard (or sample)}}{\text{OD zero standard (Bmax)}} \times 100 = \%\ \text{maximal absorbance}$$

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 O.D. values of the standards are plotted on the Y-axis versus the concentration on the X-axis. The scale of the Y-axis is logit and the X-axis is logarithmic.

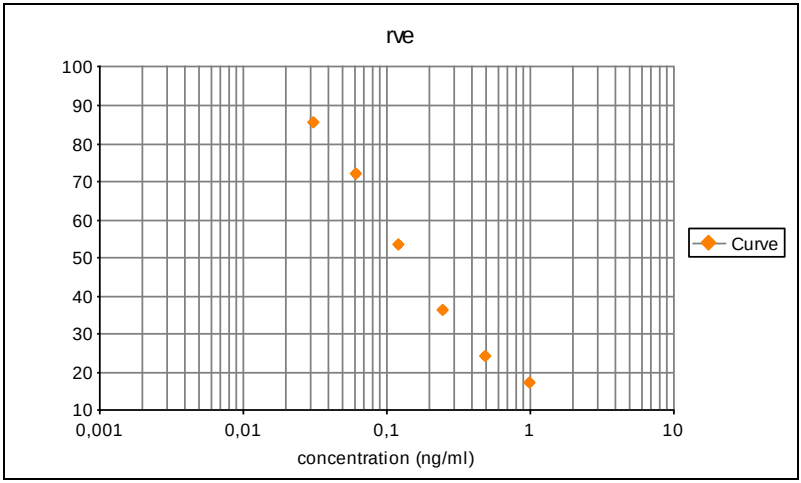


Figure 1: Example of a calibration curve

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

8.1 Tissue samples

The DES equivalents, as read from the standard curve, have to be multiplied by a factor 8 to obtain the DES content in tissue samples expressed in ppb (ng/g).

8.2 Urine samples, direct

The DES equivalents, as read from the standard curve, have to be multiplied by a factor 4 to obtain the DES content in urine samples expressed in ppb (ng/ml).

12. LITERATURE

Martin, T.G., Stob, M. Growth and carcass traits of Holstein steers, bulls, and bulls inplanted with diethylstilbestrol. Journal of Diary Science 61 (1), 132-134, 1978.

Chaud, D., Georgie, G.C. Effect of oestrogen in White Leghorn cockerells. Indian Journal of Animal Research 11 (2), 91-94, 1977.

Muir, L.A., Wien, S., Duquette, P.F., Rickes, E.L., Cordes, E.H. Effects of exogenous growth hormone and diethylstilbestrol on growth and carcass composition of growing lambs. Journal of animal science, 56 (6), 1315-1323, 1983.

Council Directive 96/22/EC of 29 April 1996 L0022.

13. ORDERING INFORMATION

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

14. REVISION HISTORY

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

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