ZERANOL ELISA

5081ZERAN[3]04.20

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

EUROPROXIMA ZERANOL ELISA

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

TABLE OF CONTENTS

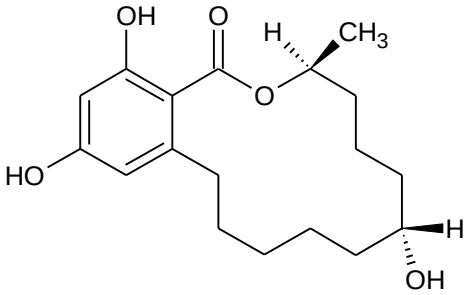
The quality management system of R-Biopharm Nederland B.V. is ISO 9001:2015 certified

5081ZERAN[3]04.20

BRIEF INFORMATION

The zeranol ELISA is a competitive enzyme immunoassay for the screening and the quantitative detection of zeranol. 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.
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 zeranol (α-zearalanol)

Zeranol (α-zearalanol) is a non-steroidal oestrogenic growth promoter that increases the live weight gain in food animals after treatment administration. It is a semi-synthetic product derived from the naturally occurring mycotoxin zearalenone. The use of zeranol for growth promotion in food animals has been banned within the EU and member states are required to monitor food-producing animals for possible abuse.
In the EU, a technical guide has been published by the Community Reference Laboratories. In this guide a recommended concentration of 1 ppb has been published for zeranol in tissue and of 2 ppb in liver as well as in urine. This means that de detection capability (ccβ) for screening methods should be lower than these values.

2. PRINCIPLE OF THE ZERANOL ELISA

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

3. SPECIFICITY AND SENSITIVITY

The zeranol ELISA utilizes antibodies raised in rabbit against protein-conjugated zeranol. The reactivity pattern of the antibody (as tested in buffer) is:

Cross-reactivity:

Zeranol (α-zearalanol)	100%
Taleranol (β-zearalanol)	133%
Zearalanone	100%
Zearalenone (F_2-mycotoxin)	75%
α-Zearalenol	90%
β-Zearalenol	70%
Diethylstilbestrol	<0.01%
Hexestrol	<0.01%
Dienestrol	<0.01%
Oestradiol-17β	<0.01%
Progesterone	<0.01%
Testosterone	<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	0.66	0.70
Liver	8.1	1.3	1.6
Urine	8.2	0.2	0.3

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.

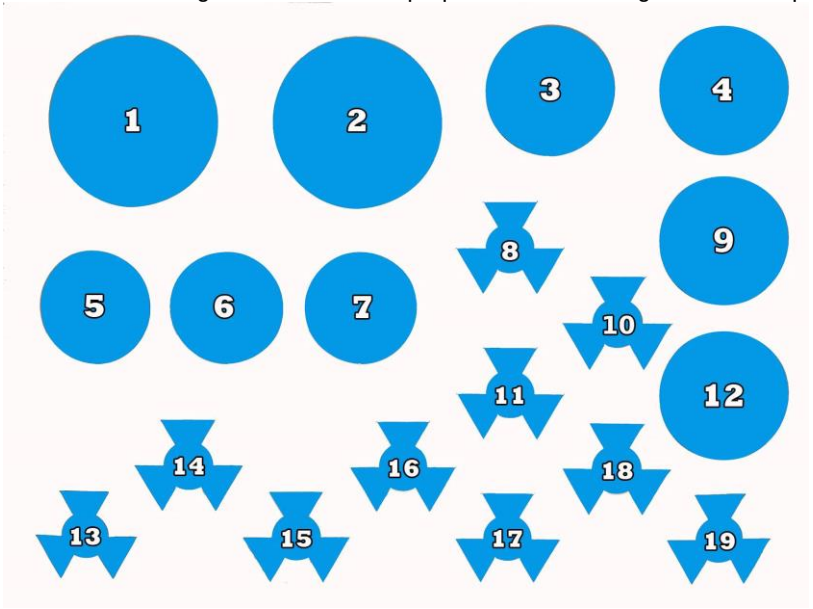
Degeneration of the reagents may have occurred when the following phenomena are observed:

- 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), precoated. Ready-to-use.

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



1. **Dilution buffer** (20 ml, 4 times concentrated)
2. **Rinsing buffer** (30 ml, 20 times concentrated)
3. **Substrate solution** (12 ml, Ready-to-use)
4. **Stop solution** (15 ml, Ready-to-use)
5. **Conjugate solution** (lyophilised)
6. **Antibody solution** (lyophilised)
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 solution** (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) **5.0 ng/ml**

6. EQUIPMENT AND MATERIALS REQUIRED BUT NOT PROVIDED

- Scales and weighing vessels
- Gloves
- Fume hood
- Homogeniser (vortex, mixer)
- Centrifuge (2500 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 (polypropylene)
- C18 cartridge (Rida®C18 column Art. n° R2002)
- Acetonitrile
- Methanol 100%
- Ethylacetate
- Distilled water
- 0.1 M Sodium-acetate buffer pH 4.8
- Helix pomatia juice 1:10 diluted in distilled water

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 Tissue/liver

- Homogenise approximately 10 g of sample
- Weigh 1 g of the homogenised sample and transfer it into a test tube
- Add 4 ml of acetonitrile
- Vortex untill the solution is a homogeneous emulsion
- Mix for 15 min head over head
- Centrifuge 2500 x g for 10 minutes
- Transfer 1 ml of the supernatant to a glass tube (volume of the tube 4 ml)
- Evaporate under a mild stream of nitrogen at 50ºC
- Reconstitute the residue with 100 µl of 100% methanol, vortex for 1 minute
- Dilute 50 µl with 450 µl dilution buffer, vortex
- Use an aliquot of 50 µl as sample in the ELISA

8.2. Urine

Hydrolysis of the urine samples

- To 1 ml of urine sample, 1 ml of 0.1 M Na-acetate buffer pH 4.8 is added
- Adjust the pH of the urine sample to a value of 4.5 – 4.8
- Add 10 µl of 1 : 10 diluted Helix pomatia juice
- Incubate either for 2 hours at 50°C, or overnight at 37°C

Activation of the C18 column

- Add 1 ml of ethylacetate
- Add 1 ml of 100% methanol
- Add 2 ml of distilled water

Note: It is important that the column is not allowed to dry completely prior to sample addition!

- Carefully bring the hydrolysed sample onto the activated column (flow speed 1 ml/minute)
- Wash the column with 1 ml of 45% methanol and allow the column to run dry
- Elute the sample with 2 ml of ethylacetate
- Evaporate the eluent under a mild stream of nitrogen at 50°C
- Dissolve the residue in 0.5 ml dilution buffer, vortex
- Use an aliquot of 50 µl 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 them at 2ºC to 8ºC for use in subsequent assays. Retain also the strip holder.

Dilution buffer
The dilution buffer is 4 times concentrated. Before dilution (20 ml buffer + 60 ml distilled water) the concentrated buffer should have reached room temperature and be thoroughly mixed. Concentrated buffer can show precipitates of the contents. Mix well before dilution with distilled water.

Conjugate solution
Reconstitute the vial of lyophilised conjugate (zeranol-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 2°C to 8°C.

Antibody solution
Reconstitute the vial of lyophilised antibodies with 4 ml dilution buffer, mix thoroughly and keep them in the dark until use.
Store the vial immediately after use in the dark at 2°C to 8°C.

Rinsing buffer
The rinsing buffer is delivered 20 times concentrated. Prepare dilutions freshly before use. Per strip 20 ml of the diluted rinsing buffer is used (1 ml concentrated rinsing buffer + 19 ml distilled water).

Substrate solution (ready to use, 12 ml)
Take care that this vial is at room temperature (keep in the dark) and mix the content before pipetting this concentrated solution (precipitates at 4°C).

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.
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 dilution buffer in duplicate (well H1, H2).
   Pipette 50 µl dilution buffer (zero standard, Bmax) in duplicate (well A1, A2).
   Pipette 50 µl of each standard solution in duplicate (well B1,2 to G1,2).
   Pipette 50 µl of each sample solution in duplicate into the remaining wells of the microtiter plate

3. Add 25 µl conjugate (zeranol-HRP) to all wells, except wells H1 and H2.

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

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

6. Incubate for 1 hour in the dark at 37ºC.

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

8. Pipette 100 µl substrate solution into each well.

9. Incubate for 30 minutes in the dark at room temperature (20°C - 25ºC).

10. Add 100 µl stop solution to each well.

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

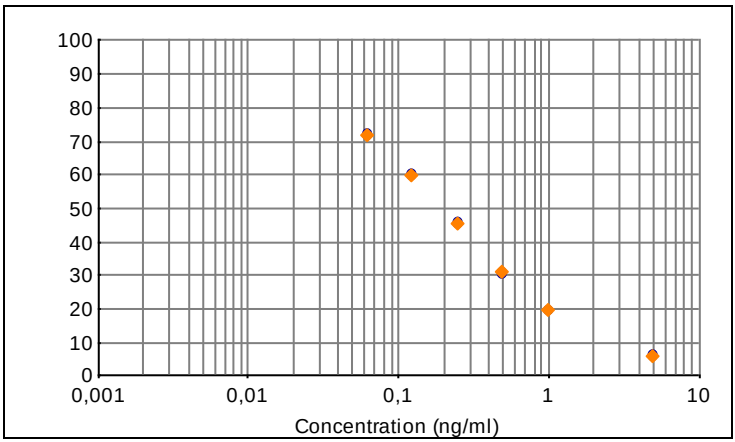


Figure 1 : Example of a calibration curve

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

8.1Tissue and liver

To obtain the zeranol content in tissue and liver samples, the calculated zeranol concentration has to be multiplied by a factor 5.

8.2 Urine

To obtain the zeranol content in urine samples, the calculated zeranol concentration has to be divided by a factor 2.

12. LITERATURE

96/22/EC. Council Directive of 29 April 1996 concerning the prohibition on the use in stockfarming of certain substances having a hormonal or thyrostatic action and of beta-agonists, and repealing Directives 81/602/EEC, 88/146/EEC and 88/299/EEC. Off. J. Eur. Communities, L125/3-9.

96/23/EC. Council Directive of 29 April 1996 on measures to monitor certain substances and residues thereof in live animals and animal products and repealing Directives 85/358/EEC and 86/469/EEC and Decisions 89/187/EEC and 91/664/EEC. Off. J. Eur. Communities, L125/10-32.

Anonymous. 2007. CRL Guidance paper of 7th December 2007. CRLs view on state of the art analytical methods for national residue control plans; [cited 2007 December 7].
Available from: http://www.rivm.nl/bibliotheek/digitaaldepot/crlguidance2007.pdf

13. ORDERING INFORMATION

For ordering the EuroProxima Zeranol ELISA kit please use catalogue code 5081ZERAN.

14. REVISION HISTORY

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

R-Biopharm Nederland B.V.
Beijerinckweg 18
6827 BN Arnhem
The Netherlands

Tel: + 31 26 3630364
www.europroxima.com
info@r-biopharm.nl