TRENBOLONE ELISA

5081TRENBO[1]05.19

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

EUROPROXIMA TRENBOLONE ELISA

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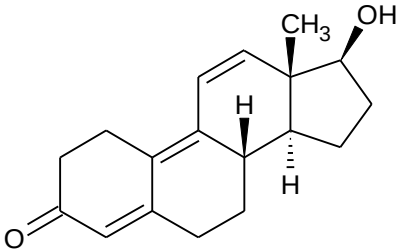
BRIEF INFORMATION

The Trenbolone ELISA is a competitive enzyme immunoassay for the screening and quantitative detection of trenbolone in urine, plasma and serum samples. 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 measured.
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 17β-trenbolone

17β-Trenbolone is an anabolic steroid used on livestock to increase muscle growth and appetite. To increase its effective half-life, trenbolone is administered as an ester conjugate such as trenbolone acetate. Plasma lipases then cleave the ester group in the bloodstream leaving free trenbolone.
Concerning EU-legislation, the relevant directive for anabolic steroids is directive 96/23/EC, i.e. on measures to monitor certain substances and residues thereof in live animals and animal products.
In the EU, a technical guide has been published by the Community Reference Laboratories. The recommended concentrations (RCs) for 17β-trenbolone in this guideline are 1 ppb for muscle and 2 ppb for urine and liver.

2. PRINCIPLE OF THE TRENBOLONE ELISA

The kit is based on a microtiter plate (12 strips, each 8 wells), precoated with sheep antibodies to rabbit IgG. Specific antibodies (rabbit anti-Trenbolone), enzyme labelled Trenbolone (enzyme conjugate) and Trenbolone standards or samples are added to the precoated wells followed by a single incubation step. The specific antibodies are bound by the immobilised anti-rabbit antibodies and at the same time Trenbolone (in the standard solution or in the urine sample) and enzyme labelled Trenbolone 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 sulfuric acid. The colour intensity is measured photometrically at 450 nm and is inversely proportional to the trenbolone concentration in the sample.

3. SPECIFICITY AND SENSITIVITY

The cross reactivity pattern of the antibody is as follows:

17β-Trenbolone	100%
Triendione (Trendione)	152%
17α-Trenbolone	54%
17β-Nortestosterone	0.2%

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)
Urine	8.1	0.5	0.7
Tissue	8.2	0.4	0.5
Liver	8.3	0.6	0.7

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.
- 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.

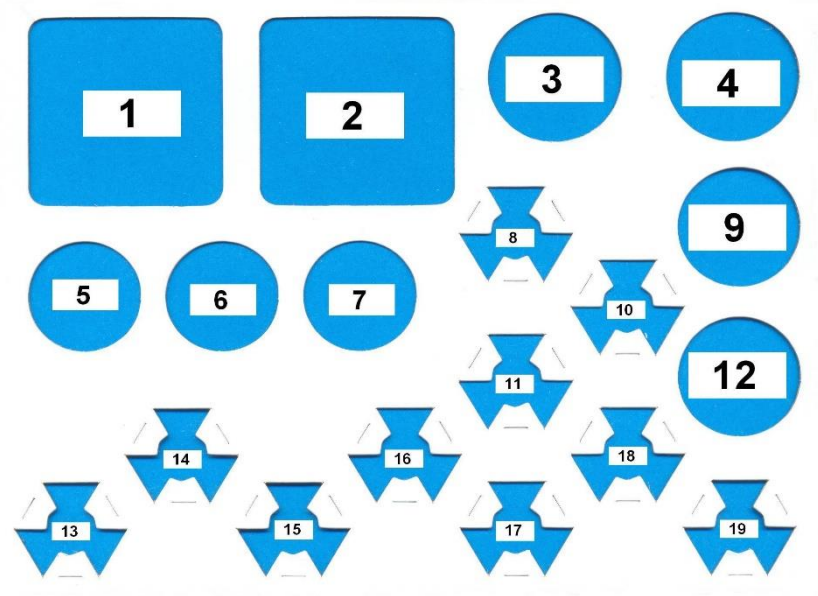
Degeneration of the reagents may have occurred when the following phenomena are observed:
- A blue colouring of the chromogen solution before transferring it into the wells.
- A weak or no colour reaction in the zero standard wells (E450nm < 0.8).

5. KIT CONTENTS

Manual

One sealed 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 9.



1. **Dilution buffer** (20 ml)
2. **Rinsing buffer** (30 ml, 20x concentrated)
3. **Substrate** (12 ml, ready-to-use)
4. **Stop solution** (15 ml, ready-to-use)
5. **Conjugate** (lyophilized, blue cap)
6. **Antibody** (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) **0.125 ng/ml**
15. **Standard solution 2** (1ml, ready-to-use) **0.25 ng/ml**
16. **Standard solution 3** (1ml, ready-to-use) **0.5 ng/ml**
17. **Standard solution 4** (1ml, ready-to-use) **1.0 ng/ml**
18. **Standard solution 5** (1ml, ready-to-use) **5.0 ng/ml**
19. **Standard solution 6** (1ml, ready-to-use) **10.0 ng/ml**

6. EGUIPMENT 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
- Sodium acetate
- Rida®C18 columns, article n° R2002
- Helix pomatia juice (Merck Art. No.: 4114)
- Tert-butylmethylether

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 Urine samples

Hydrolysis

- To 1 ml of urine, 1 ml of 0.1 M acetate buffer*, pH 4.8 is added
- The pH of the urine samples is checked (pH between 4.5 and 4.8)
- 10 µl Helix pomatia juice, 1:10 diluted in distilled water, is added
- After an incubation of 2 hours at 50ºC or alternatively overnight at 37ºC the solid phase extraction with the RIDA® C18 column is performed.

Activation of the cartridges
- Wet the sorbent by adding successively
- 1 ml ethyl acetate
- 1 ml 100% methanol
- 2 ml distilled water

Note: It is important that the disk is not allowed to dry completely prior to sample addition! If the disk has become dry, repeat the conditioning procedure.
- Add the hydrolyzed urine sample
- Carefully transfer the sample into the cartridge
- Pass the sample through the disk using vacuum

Washing procedure
- add 1.0 ml 45% methanol and allow disk to become dry

Elution
- Add 2 ml ethyl acetate
- Evaporate the eluent to dryness at 30ºC under a mild stream of nitrogen
- Dissolve the residue in 0.5 ml of Phosphate Buffer/Tween (see 9)
- Two times 50 µl of this solution is used in the ELISA

8.2 Tissue
- Remove fat and grind sample
- Homogenize 10 g of ground sample with 10 ml of 67 mM PBS buffer (see 9) shake for 5 min
- Homogenize 10 g of ground sample with 10 ml of 67 mM PBS buffer (see 9) shake for 5 min
- Mix 2 g of the homogenized sample with 5 ml of *tert*-Butyl methyl ether in a centrifugal screw cap vial and shake vigorously for 30 – 60 min
- Centrifuge: 10 min / 3000g / 10 – 15 °C
- Transfer the supernatant into another glass vial with screw cap
- Repeat the extraction procedure with another 5 ml of *tert*-Butyl methyl ether
- Evaporate the combined ether layers and then dissolve in 1 ml of methanol (80%)
- Dilute the methanolic solution with 2 ml of 20 mM PBS (see 9)
- This solution is further purified by means of RIDA® C18 column

- Rinse the column with 3 ml MeOH (100%), flow rate: 1 drop per sec
- Equilibrate the column with 2 ml 20 mM PBS (see 9)
- Apply sample (volume after hydrolyzation or extraction as described)
- Rinse the column with 2 ml MeOH (10%)
- Ensure that all liquid is removed from the column by pressing air or N_2 through the column
- Elute slowly with 1 ml MeOH (80%), flow rate: 15 drops per min
- Dilute eluate 1:2 (1 + 1) with distilled water
- Use 50 µl per well in the test

8.3 Liver

- Homogenize 1 g of liver with 2 ml of 0.5 M sodium acetate buffer (see 9)
- Add 8 µl of glucuronidase/arylsulfatase of *Helix pomatia* (see 9)
- For hydrolyzation incubate the solution for 3 h at 37 °C, or alternatively overnight at room temperature (20 – 25 °C
- As described in section 8.2, the hydrolyzed product is further purified, beginning with the *tert*-Butyl methyl ether extraction

9. PREPARATION OF REAGENTS

Before starting the test, allow the reagents to come to ambient temperature. Any reagents not used should be put back into storage immediately at 2°C to 8°C.

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.

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) precipitates at 4ºC. Take care that this vial is at 20°C to 25°C (keep in the dark) and mix the content before pipetting in the wells.

Conjugate solution
Reconstitute the vial of lyophilized conjugate (TRENBO-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 lyophilized 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 2°C to 8°C.

Note: For prolonged storage of standard solutions, antibody and conjugate store aliquots at –20ºC.

Phosphate buffer pH 7.4
Dissolve in 1000 ml distilled water.

Na_2HPO_4	1.15 g
KH_2PO_4	0.2 g
KCl	0.2 g
NaCl	30.0 g
Tween 80	0.15 ml
BSA (Sigma A7030)	10 g

67 mM PBS buffer
pH 7.2 – 7.5

$NaH_2PO_4 \times H_2O$	1.8 g
$Na_2HPO_4 \times 2\ H_2O$	9.61 g
NaCl	9.0 g

fill up to 1000 ml with distilled water

20 mM PBS buffer
pH 7.2 – 7.5
$NaH_2PO_4 \times H_2O$ 0.55 g
$Na_2HPO_4 \times 2\ H_2O$ 2.85 g
NaCl 9.0 g
fill up to 1000 ml with distilled water

0.1 M acetate buffer
pH 4.8
Sodium acetate 8.2 g
Fill up to 1000 ml with distilled water, adjust to pH 4.8 with acetic acid

0.5 M sodium acetate buffer
pH 4.8
sodium acetate 41.0 g
fill up to 1000 ml with distilled water, adjust to pH 4.8 with 20 % acetic acid

10. ASSAY PROCEDURE

Rinsing protocol

In ELISA's, 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 (steps 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 (Sample preparation) and prepare reagents according to Chapter 9. All standards and samples should be simultaneously tested in duplicate.

2. Pipette 100 µl of zero standard in duplicate (well H1, H2, blank).
   Pipette 50 µl of zero standard (Bmax) in duplicate (well A1, A2).
   Pipette 50 µl of standard solution in duplicate (well B1, B2 to G1, G2).

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

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

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

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

7. Incubate for 1 hour in the dark at 20ºC - 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 20ºC - 25ºC.

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

12. Read the absorbance 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 absorption 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.

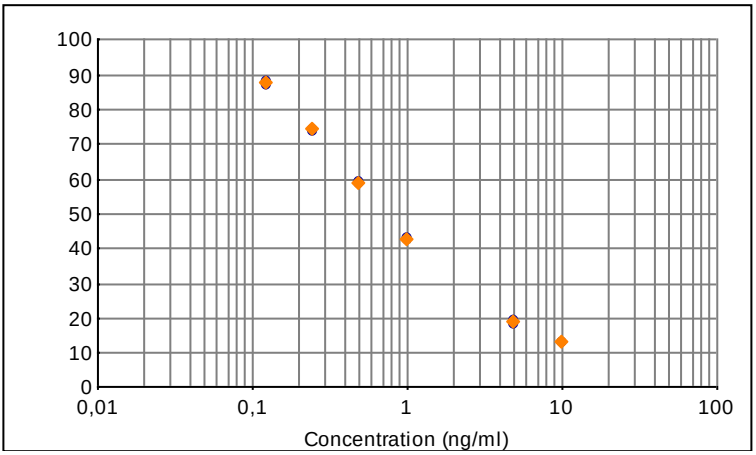


Figure 1: Example of a calibration curve

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

8.1 Urine samples
The Trenbolone equivalents, as read from the standard curve, have to be divided by a factor 2, to obtain the trenbolone content in urine samples.

8.2 Tissue samples
The Trenbolone equivalents, as read from the standard curve, have to be multiplied by a factor 2, to obtain the trenbolone content in tissue samples.

8.3 Liver samples
The Trenbolone equivalent, as read form the standard curve, have to be multiplied by a factor 2, to obtain the trenbolone content in tissue samples.

12. LITERATURE

Council Directive 96/23/EC of 29 April 1996. Off. J. European Comm. L125 (1996) 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. Available from: http://www.rivm.nl/bibliotheek/digitaaldepot/crlguidance2007.pdf

13. ORDERING INFORMATION

For ordering the Trenbolone ELISA kit, please use catalogue code 5081TRENBO.

14. REVISION HISTORY

Not applicable.

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