10. LITERATURE

DEGAND, G., SCHMITZ, P. and MAGHUIN-ROGISTER, G..
Enzyme immunoassay screening procedure for the synthetic anabolic estrogens and androgens diethylstilbestrol, nortestosterone, methyltestosterone and trenbolone in bovine urine.
Journal of Chromatography, 489 (1989) 235-243

11. ORDERING INFORMATION

For ordering the Trenbolone EIA kit, please use cat. code 5081TREN1p.

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TRENBOLONE EIA

A microtiter plate based enzyme immunoassay for the screening on the presence of Trenbolone in biological matrices

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BRIEF INFORMATION

The Trenbolone-EIA is a competitive enzyme immunoassay for the screening of urine samples on the presence of this anabolic steroid and its main metabolites. This EIA system uses antibodies raised against protein conjugated Trenbolone. With this EIA-kit 96 analyses can be performed. Samples and standards are measured in duplicate which means that in total 40 samples can be analysed. The EIA kit contains all the reagents, including standards to perform the test. Chemicals for the preparation of extracts are not included.

1. PRINCIPLE OF THE TRENBOLONE EIA

The microtiter plate based EIA kit consists of 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 sulphuric acid. The colour intensity is measured photometrically at 450 nm and is inversely proportional to the steroid concentration in the standard solution or the sample.

2. SPECIFICITY AND SENSITIVITY

The Trenbolone EIA utilizes antibodies raised in rabbits against protein conjugated Trenbolone. As shown in Figure 1 (page 7), the calibration curve is visually linear between 0.125 and 10 ng/ml of Trenbolone.

Cross-reactions:

17β-Trenbolone	100	%
17α-Trenbolone	63	%
Altrenogest	38	%
19β-Nortestosterone	2	%
Testosterone	< 0.01	%
Progesterone	< 0.01	%
17β-Oestradiol	< 0.01	%

3. HANDLING AND STORAGE

- Store the kit at +2ºC to +8ºC in a dark place.
- After the expiry date (see kit label) has passed, it is no longer possible to accept any further quality guarantee.
- It is advised to unpack the sealed microtiter plate, reconstitute or dilute the kit components, immediately before use.
- After the lyophilised kit components have been reconstituted, these components are only guaranteed for 1 week (stored at +2ºC to +8ºC in the dark).

9. INTERPRETATION OF RESULTS

Subtract the mean OD value of the wells A1 and A2 from the individual OD of the wells containing the standards and the samples. The OD values of the standards and the samples (mean values of the duplicates) are divided by the mean OD value of the zero standard (wells B1 and B2) and multiplied by 100. The zero standard is thus equal to 100% (maximal OD) and the other OD values are quoted in percentages of the maximal OD.

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

Calibration curve:

The values (% maximal OD) calculated for the standards are plotted (on the linear Y-axis) versus the equivalent concentration (ng/ml) on a logarithmic X-axis. The calibration curve should be virtually linear in the 0.125-10 ng/ml range.

Urine samples:

The calculated Trenbolone equivalents have to be divided by a factor 2. The amount of Trenbolone in the samples is expressed as Trenbolone equivalents. The Trenbolone equivalents in the extracts (ng/ml) corresponding to the % maximal OD of each extract can be read from the calibration curve.

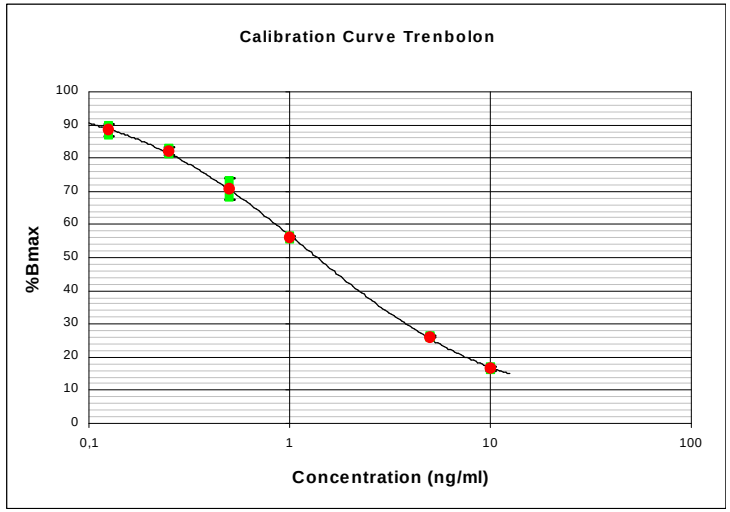

Figure 1: Example of a calibration curve

8. ASSAY PROCEDURE

- Rinsing protocol

In EIA's, between each immunological incubation step, unbound components have to be removed efficiently. This is achieved by appropriate rinsing. It should be clear that each rinsing procedure must be carried out with care to guarantee inter- and intra-assay results. Basically, manual rinsing or rinsing with automatic plate washing equipment can be done as follows:

Manual rinsing

1. Empty the content of each well by turning the microtiter plate upside down followed by a firm short vertical movement.
2. Fill all the wells to the rim (300 µl) with washing 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. Take care that none of the wells dry out before the next reagent is dispensed.

Rinsing with automatic microtiter plate washing equipment
When using automatic plate washing equipment, check that all wells can be aspirated completely, that the washing solution in correctly 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 6 (Sample treatment) and prepare reagents according to Chapter 7. All standards and samples should be simultaneously tested in duplicate.

   Microtitre plate is ready for use, do not wash.

2. Pipette 100 µl of dilution buffer in duplicate (well A1, A2).
   Pipette 50 µl of dilution buffer in duplicate (well B1, B2).
   Pipette 50 µl of standard solution in duplicate (well C1, C2 to, H1, H2).
   Pipette 50 µl of each sample solution in duplicate to the remaining wells of the microtiter plate (40 samples, 80 wells).
3. Pipette 25 µl of conjugate (Trenbolone-HRPO) to all wells, except wells A1 and A2.
4. Pipette 25 µl antibody solution to all wells, except wells A1 and A2.
5. Seal the microtiter plate and shake the plate for 1 min.
6. Incubate for 1 hour in the dark at room temperature (20-25ºC).
7. Discard the solution from the microtiter plate and wash 3 times with rinsing buffer.
8. Pipette 100 µl of substrate solution into each well.
9. Incubate 30 min. at room temperature (20-25ºC) in the dark.
10. Add 100 µl of stop solution to each well.
11. Read the OD values immediately at 450 nm.

It is advised to store the reconstituted components in small aliquots at –20ºC.

- Any direct action of light on the chromogen solution should be avoided.

If the following phenomena are observed, this may indicate a degradation of the reagents:

- A blue colouring of the chromogen solution before putting it into the wells,
- A weak or absent colour reaction of the first standard (0-standard) (E450nm < 0.8).

4. KIT CONTENTS

The reagents included in the kit are sufficient to carry out at least 96 analyses (including standard analyses). Each standard and sample is analysed in duplicate.

Contents EIA-kit:

- 1 sealed (96-wells) Microtiter plate (12 strips, 8 wells each), coated with antibodies to rabbit IgG. Ready to use, do not wash before use.
- 6 vials (0.5 ml each) containing ready to use standard solutions of Trenbolone (10; 5; 1; 0.5; 0.25 and 0.125 ng/ml),
- 1 vial containing lyophilised conjugate (peroxidase conjugated to Trenbolone, blue cap),
- 1 vial containing lyophilised antibodies (anti-Trenbolone; gold cap),
- 1 vial containing the Substrate solution. Ready to use (12 ml).
- 1 vial containing dilution buffer pH 7. Ready to use (white cap, 20 ml).
- 1 vial containing 10 times concentrated rinsing buffer (60 ml).
- 1 vial containing stop solution. Ready to use 15 ml (red cap)

5. PRECAUTIONS

- The stop solution contains 0.5 M sulphuric acid. Do not allow the reagent to get into contact with the skin and / or eyes.
- 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; observe care when handling the substrate.

- Do not use components past expiration date and do not intermix components from different serial lots.

- Each well is ultimately used as an optical cuvette. Therefore, do not touch the under surface of the wells, prevent damage and dirt.

- Optimal results will be obtained by strict adherence to this protocol. Careful pipetting and washing throughout this procedure are necessary to obtain optimal results.

6. SAMPLE PREPARATION

This test can be used for measuring Trenbolone in biological matrices Alternative methods may be used. A method for analysis of Trenbolone in urine samples has been validated.

6,1 Hydrolysis of urine samples.

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) and 10 µl Helix pomatia juice (Merck art. no. 4114), 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 and clean-up step is performed.

6,2 Solid phase extraction.

Empore Extraction Disk Cartridges; Octadecyl (C18) SD, Standard density optimised for biological matrices, 7mm/3ml cartridges. Catalogue No. 1214-4002. VARIAN, sample preparation products. Activation c.q. condition of the cartridges: Wet the sorbent by adding successively 1 ml ethyl acetate, 1 ml 100% methanol and 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 hydrolysed sample (1 ml of urine + 1 ml acetate buffer): Carefully transfer the sample into the Empore disk extraction 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 and dissolve in 0.5 ml of Phosphate Buffer/Tween (See Chapter 7). Two times 50 µl of this solution is used in the EIA.

7. 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 +2ºC to +8ºC.
Return unused strips into plastic bag, seal bag with desiccant and store at +2ºC to +8ºC for use in subsequent assays. Retain also the strip holder.

- Rinsing buffer

  The rinsing buffer is delivered 10 times concentrated. Prepare dilutions freshly before use. Per strip 40 ml of diluted rinsing buffer is used (4 ml concentrated rinsing buffer + 36 ml distilled water).

- Substrate solution
  The substrate solution (ready to use) precipitates at 4ºC. Take care that this vial is at room temperature (keep in the dark) and mix the content before pipetting in the wells.

- Conjugate solution

  Reconstitute the vial of lyophilised conjugate (TREN-HRPO) with 3 ml dilution buffer, mix thoroughly and keep in the dark until use. Store the vial immediately after use in the dark at +2ºC to +8ºC.

- Antibody solution

  Reconstitute the vial of lyophilised antibodies with 3 ml 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 of standard solutions, antibody and conjugate store aliquots at –20 ºC.**

- 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 20	0.15 ml
BSA (Sigma A7030)	10 g