19-NORTESTOSTERONE-ELISA

A competitive enzyme immunoassay for
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BRIEF INFORMATION

The 19-Nortestosterone ELISA is a competitive enzyme immunoassay for the concentration of 19-nortestosterone. 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 analyzed.
The ELISA kit contains all reagents to perform the assay. Reagents for sample preparation are not included in the kit.

1. INTRODUCTION

Anabolic preparations are attractive means of improving the growth rate and feed conversion of animals in livestock breeding [1]. In all European Community countries however, the use of growth promoters has been banned since 1986 [2]. The anabolic steroid 19-nortestosterone is one of the most frequently found growth promoters. The European Community allows a limit of 2 ng/ml 19-nortestosterone in bile, faeces and urine.
In most EC countries urine is chosen as the matrix to screen for the presence of growth promoters. In the urine of most species anabolics and their (hydroxy) metabolites are mainly excreted as conjugates of glucuronic and sulphuric acids. For the determination of the analytes in urine from treated animals, (enzymatic) hydrolysis of the conjugates to free steroids is necessary.
With this ELISA it is possible to screen a large amount of urine samples in a short time for the presence of 17β-19-nortestosterone and its major metabolites (17α-19-nortestosterone and 19-norandrostendione).
To reduce the influence of other urine compounds, a solid-phase clean-up is recommended.
This test makes no distinction between the anabolic steroid and its metabolites described before and can therefore only be used as a screening method. For the confirmation of the presence and the identification of the specific steroid, GC-MS based methods are recommended.

NOTE: Endogenous 19-nortestosterone occurs in urine samples of male pigs (boars) [3] and horses [4-5].

2. PRINCIPLE OF THE 19-NORTESTOSTERONE ELISA

The microtiter plate based ELISA kit consists of one precoated plate (12 strips, 8 wells each) with sheep antibodies to rabbit IgG. In one incubation step, specific antibodies (rabbit anti-17β-19-nortestosterone), enzyme labelled nortestosterone (enzyme conjugate) and nortestosterone standards or samples are added to the precoated wells. The specific antibodies are bound by the immobilised anti-rabbit antibodies and at the same time nortestosterone (in the standard solution or in the samples) and enzyme labelled nortestosterone 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 visualised 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 steroid concentration in the standard solution or the sample.

3. SPECIFICITY AND SENSITIVITY

The 19-nortestosterone ELISA utilizes antibodies raised in rabbits against protein conjugated 17β-19-nortestosterone.

Cross-reactivity:	17β-19-nortestosterone	100%
	17α-19-nortestosterone	50%
	nortestosterone-sulphate	100%
	19-nor-4-androstene-3, 17-dione	240%
	norgestrel	16%
	norethindrone	38%
	19-nor-4-androstene-6β-ol-3, 17-dione	8%
	19-nor-4-androstene-15α-ol-3, 17-dione	6%
	17β-trenbolone	5%
	progesterone	0.3%
	5α-estrane-3β, 17α-diol	0.2%
	5α-dihydrotestosterone	0.2%
	17α-testosterone	0.1%
	17β-testosterone	< 0.1%
	methyltestosterone	< 0.1%
	5α-androstane-3α, 17β-diol	< 0.1%
	17α-estradiol	< 0.1%
	17β-estradiol	< 0.1%
	estrone	< 0.1%
	estriol	< 0.1%
	ethinylestradiol	< 0.1%

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

12. LITERATURE:

1. P.L.M. Berende and E.J. Ruitenberg, in L.J. Peel and D.E. Tribs (Editors), Domestication, Conservation and Use of Animal Resources, Elsevier, Amsterdam, 1983, pp. 191-233.
2. Council of the European Communities, Council Directive 86/469/EEC of 16 September 1986, Off. J. Eur. Commun., L275 (1986) 36.
3. W. Haasnoot et al., Archiv Lebensmittelhyg., 41 (1990) 131-138 .
4. E.F. Benoit et al., Ann. Rech. Vet., 16 (1985) 379-383.
5. P. Silberzahn et al., Endocrinology, 17 (1985) 2176-2181.

13. ORDERING INFORMATION

For ordering the Nortestosterone ELISA kit, please use cat. code 5081NOR.

14. LAST MUTATIONS

Milk samples treatment is added.
Pipette schedule of the standard curve is adapted.

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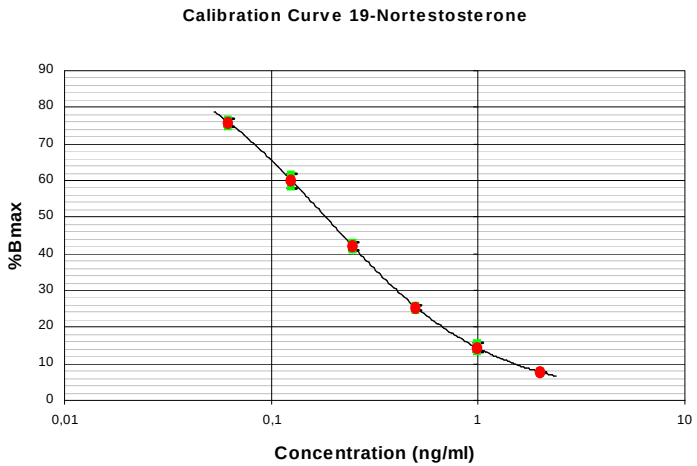


Figure 1 : Example of a calibration curve

8.1 Urine samples:
The nortestosterone equivalent read from the calibration curve have to be multiplied by a factor 10 to obtain the nortestosterone equivalents in the urine samples (ng/ml).

Remark: Urine from treated animals contains higher concentrations of metabolites (about 10 times for 17α-19-nortestosterone) compared to the parent compound 17β-19-nortestosterone. This ELISA detects both, the parent compound and the major metabolites.

8.2 Milk samples
The nortestosterone equivalents found in the milk have to be multiplied by a factor 5 to obtain ng/ml nortestosterone equivalents in the sample.

"Positive" samples found with this ELISA have to be confirmed on the presence of nortestosterone residues by a more specific method (preferably a GC-MS based method).

Matrix	Procedure	LOD (ppb)
Urine	8.1	0.5
Milk	8.2	0.5

4. HANDLING AND STORAGE

- Store the kit at + 2ºC to + 8ºC in a dark place. For repeated use store the 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 kit components have been reconstituted, these components are only guaranteed for 1 week (stored at + 2ºC to + 8ºC in the dark). 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.

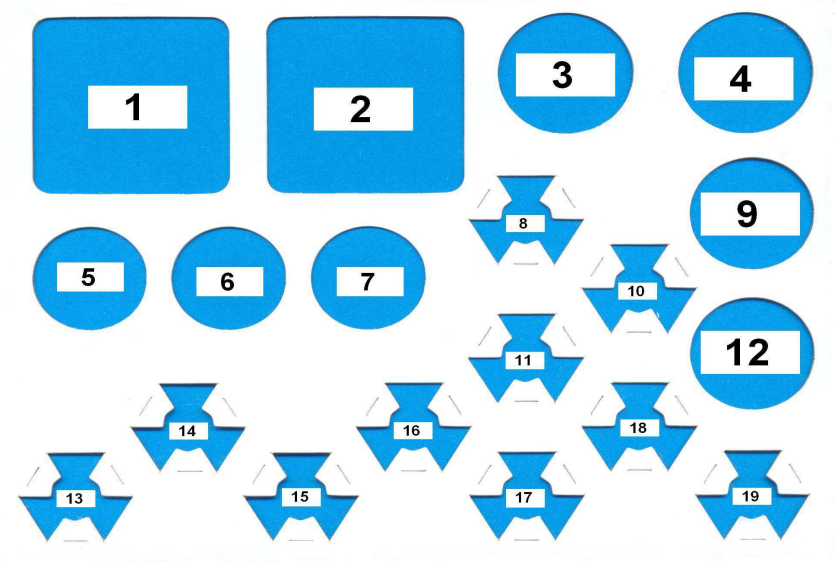
Degeneration of the reagents may have occurred when the following phenomena are observed:
- A blue colouring of the substrate solution before transferring it into the wells.
- A weak or absent colour reaction of the zero standard (Bmax, E450nm < 0.8).

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)
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** (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**

7. Incubate for 1 hour in the dark at 4ºC (2ºC - 8ºC).
8. Discard the solution from the microtiter plate and wash 3 times with rinsing buffer.
9. Pipette 100 µl substrate solution into each well.
10. Incubate 30 minutes in the dark at room temperature (20ºC - 25ºC).
11. Pipette 100 µl 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 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.

$$\frac{\text{O.D. standard (or sample)}}{\text{O.D. zero standard/Bmax}} \times 100\% = \% \text{ maximal O.D.}$$

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.

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 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 from 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 wash equipment, check that all wells can be aspirated completely, that the rinsing solution in 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 zero standard (Bmax) in duplicate (wells A1, A2).
   Pipette 50 µl of each standard dilutions in duplicate (wells B1, 2 to G1,2).

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

4. Pipette 25 µl of Enzyme conjugate solution (NOR-HRP) to all wells, except wells H1 and H2.

5. Add 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.

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 (Greiner, polypropylene)
- C18 column 7 mm/3 ml. Catalogue number 1214-4002 Agilent Technologies
- Glucoronidase/arylsulfatase of Helix pomotia (Merck 1.04114.0002)
- Acetonitrile
- Methanol 100%

7. PRECAUTIONS

- 19-Nortestosterone is toxic compound. Avoid contact with mouth and skin. Be aware that 19-nortestosterone 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 PREPARATIONS

This test can be used for measuring Nortestosterone in biological matrices. Alternative methods may be used.

8.1 Hydrolysis of the 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, 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.

Solid phase extraction

Activation of the cartridges: Wet the sorbent by adding successively 1 ml ethylacetate, 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 ethylacetate.

Evaporate the eluent to dryness at 30ºC under a slow flow of nitrogen and dissolve in 0.5 ml of sample dilution buffer/Tween (SDBT+) (see chapter 9). 50 µl of this solution is diluted with 950 µl SDBT+ and 2 x 50µl is used in the ELISA.

8.2 Milk samples

Note: For undiluted milk samples the matrix effect is a critical factor. The pH of the sample is a potential pitfall. Sour milk disturbs the ELISA, neutralization of the pH is essential.

- To 50 µl homogenized milk add 200 µl of dilution buffer and mix thoroughly
- Use 50 µl of the mixture 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 - 8ºC. 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.

Rinsing buffer
The rinsing buffer is delivered 20 times 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 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 solution (precipitates at 4ºC).

Conjugate solution
Reconstitute the vial of lyophilized conjugate (NOR-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 +2ºC to +8ºC. For prolonged storage aliquot and freeze at -20ºC.

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 +2ºC to +8ºC. For prolonged storage aliquot and freeze at -20ºC.

Sample dilution buffer/Tween (SDBT+)

Dissolve in 1000 ml distilled 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 A7030)	10	g