19-NORTESTOSTERONE-ELISA

5081NOR[11]05.20

A competitive enzyme immunoassay for
screening and quantitative analysis of
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EUROPROXIMA 19-NORTESTOSTERONE-ELISA

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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. In all European Community countries however, the use of growth promoters has been banned since 1986. 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.
Endogenous 19-nortestosterone occurs in urine of male pigs and horses.
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.

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 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) is determined under optimal conditions. Cut-off criteria need critical consideration.

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

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.

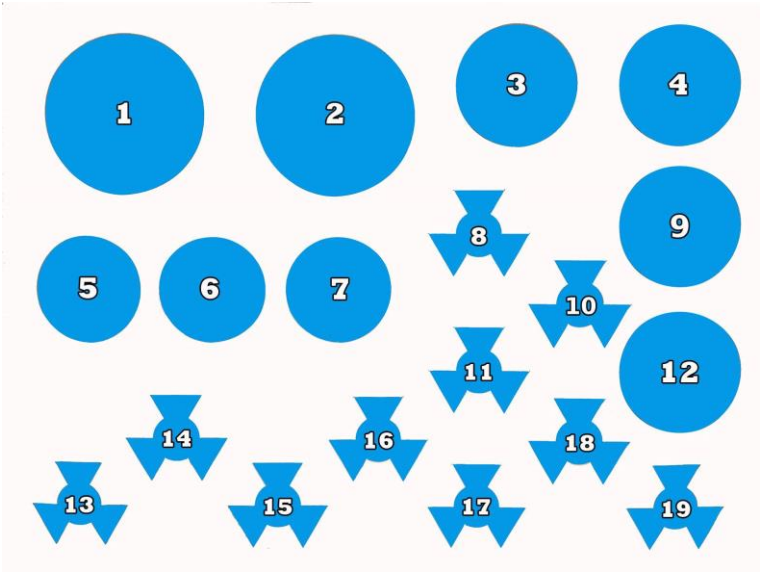
Degeneration of the reagents may have occurred when the following phenomena are observed:
- A blue colouring of the chromogen solution before transferring it into the wells.
- A weak or no colour reaction in the zero standard wells (E450nm < 0.8).

5. KIT CONTENTS

Manual

One sealed (96-wells) microtiter plate (12 strips, 8 wells each), coated with 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, ready-to-use)
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 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) **2.0 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 column 7 mm/3 ml.
- Glucoronidase/arylsulfatase of Helix pomatia
- Methanol 100%
- Ethylacetate
- 0.1M acetate buffer
- Distilled water
- Disodium hydrogen phosphate Na_2HPO_4
- Potassium dihydrogen phosphate KH_2PO_4
- Potassium chloride KCl
- Sodium Chloride NaCl
- Tween 80
- BSA (Sigma A7030)

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 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 is performed.

Solid phase extraction

Activation of the column:
Wet the sorbent by adding successively
1 ml ethylacetate,
1 ml 100% methanol
2 ml distilled water

Note: It is important that the column is not allowed to dry completely prior to sample addition! If the column has become dry, repeat the conditioning procedure.

Add hydrolysed sample.
Carefully transfer the sample into the column extraction cartridge. Pass the sample through the column using vacuum.

Washing procedure:
add 1.0 ml 45% methanol and allow column 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 (see chapter 9).

50 µl of this solution is diluted with 950 µl sample dilution buffer.
Use 50µl of this solution in the ELISA test.

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 this solution 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 20 ml of diluted rinsing buffer is used (1 ml concentrated rinsing buffer + 19 ml distilled water).

Substrate solution
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.

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.

Sample dilution

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

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 and prepare reagents according to chapter 9.

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.

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.

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

Calibration curve:
The values (percentage 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.

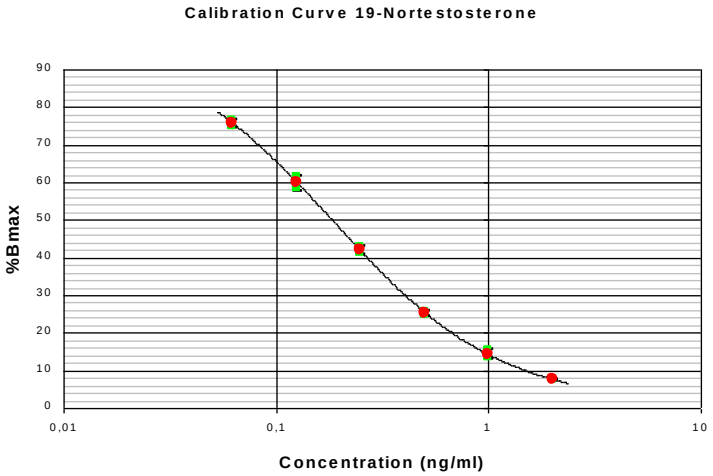


Figure 1 : Example of a calibration curve

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).

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.

12. LITERATURE:

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13. ORDERING INFORMATION

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

14. REVISION HISTORY

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

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