STANOZOLOL ELISA

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

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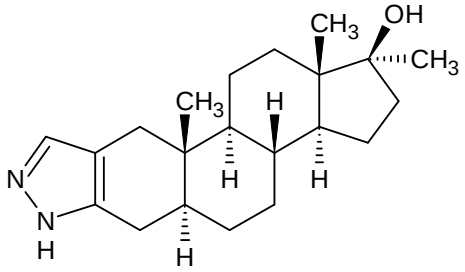
BRIEF INFORMATION

The Stanozolol ELISA is a competitive enzyme immunoassay for the screening of cattle urine or faeces samples. The test is based on antibodies directed against stanozolol. The ELISA kit contains a 96 well microtiter plate as well as all essential reagents including ready-to-use standards to perform the test. Methods for a fast and efficient extraction of stanozolol from different matrices are included in the kit manual.

1. INTRODUCTION



Chemical structure of stanozolol

Stanozolol is a synthetic anabolic steroid derived from dihydrotestosterone. In contrast to most injectable anabolic steroids, stanozolol is not esterified and is sold either as an aqueous suspension or in oral tablet form. Like other anabolic steroids, stanozolol increases protein-synthesis within cells, which results in the build up of cellular tissue, especially in muscles.

Concerning EU-legislation, the relevant directive for anabolic steroids is directive 96/23/EC [1], 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 [2]. The recommended concentrations (RCs) for stanozolol in this guideline are 1 ppb for muscle and 2 ppb for urine and liver.

2. PRINCIPLE OF THE STANOZOLOL ELISA

The kit is based on a microtiter plate (12 strips, each 8 wells), precoated with sheep antibody to rabbit IgG. In one incubation step, specific antibodies (rabbit anti-STAN), enzyme labelled stanozolol and stanozolol standards or sample are added to the precoated wells. The specific antibodies are bound by the immobilized rabbit antibodies and at the same time free stanozolol (in the standard solution or in the sample) and enzyme labelled stanozolol compete for the specific antibody binding sites (competitive enzyme immunoassay).
After an incubation of 30 minutes, the non-bound (enzyme labelled) reagents are removed in a washing step. The amount of bound enzym conjugate is visualized by the addition of a substrate/chromogen solution (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 stanozolol concentration in the sample.

3. SPECIFICITY AND SENSITIVITY

The Stanozolol ELISA utilizes antibodies raised in rabbits against protein conjugated stanozolol. The reactivity pattern of the antibody is:

Cross-reactivity:	stanozolol	100%
	16β-hydroxy stanozolol	100%
	4β-hydroxy stanozolol	0.30%
	3´ hydroxy stanozolol	< 0.01%
	methyltestosterone	< 0.01%
	testosterone	< 0.01%

The CCβ is determined under optimal conditions.

Matrix	Procedure	CCβ (ppb)
Urine	8.1	1
Faeces	8.2	1

8.1.2 Urine (extraction method)
The stanozolol equivalents, as read from the standard curve, have to be multiplied by a factor 2. To obtain the stanozolol content in urine samples.

8.2 Faeces
The stanozolol. equivalents, as read from the standard curve, have to be multiplied by a factor 2. To obtain the stanozolol content in faeces samples.

12. LITERATURE

1. Council Directive 96/23/EC of 29 April 1996. Off. J. European Comm. L125 (1996) 10-32.
2. 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 stanozolol kit, please use cat. code 5081STAN.

14. LAST MUTATIONS

Recipe PBST is added.
Chapter 1 Introduction is revised.
Added chapters:
Chapter 6 Equipment and materials required but not provided
Chapter 7 Precautions
Chapter 12 Literature
Updated lay-out

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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 dilution buffer (Bmax, wells A1 and A2) and multiplied by 100. The dilution buffer (Bmax) is thus made equal to 100% (maximal absorbance) and the other O.D. values are quoted in percentages of the maximal absorbance.

O.D. standard (or sample)
----------x 100% = % maximal absorbance
O.D. dilution buffer (Bmax)

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.

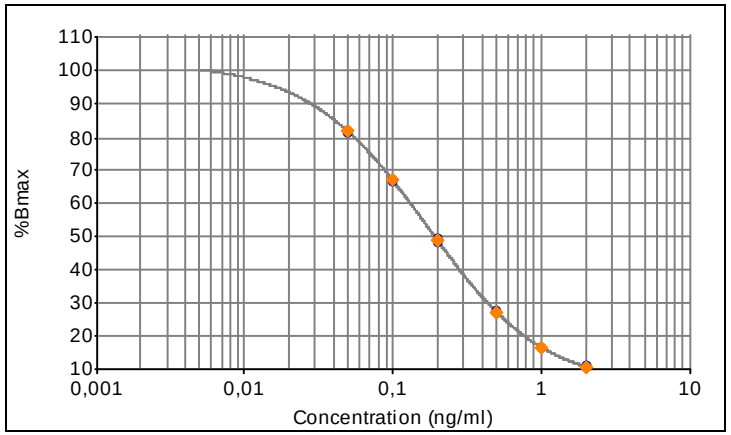


Figure 1 : Example of a calibration curve

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

8.1.1 urine (direct method)
The stanozolol equivalents, as read from the standard curve, have to be multiplied by a factor 5. To obtain the stanozolol content in urine samples.

4. HANDLING AND STORAGE

- Kit and kit components are stored in a refrigerator (2°C to 8°C) before and immediately after use.
- 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 at ambient (room) temperature before use.
- Avoid condensation in the wells of the plate. Bring the sealed plate at ambient temperature before opening the plate sealing.
- Any direct action of light on the chromogen solution should be avoided.

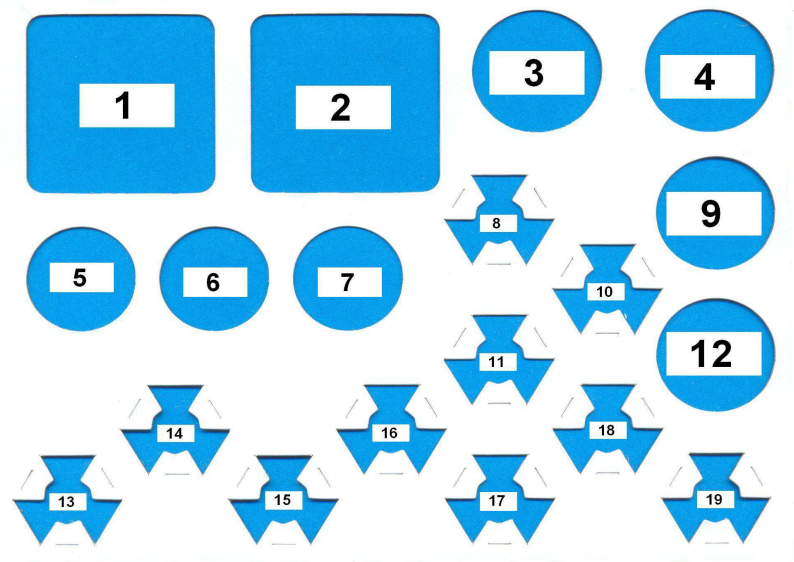
Degeneration of the reagents may have occurred when the following phenomena are observed:
- A blue colouring of the chromogen solution before transferring it into the wells.
- A weak or absent colour reaction of the maximum binding (zero standard) (E450nm < 0.8).

5. KIT CONTENTS

Manual

One sealed microtiter plate (12 strips, 8 wells each), coated with antibodies to rabbit IgG. Plate is ready-to-use.

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



1. **Dilution buffer** (30 ml, 10x concentrated)
2. **Rinsing buffer** (30 ml, 20x concentrated)
3. **Substrate solution** (12 ml, ready-to-use)
4. **Stop solution** (15 ml, ready-to-use)
5. **Anti-stanozolol antibody** (lyophilized, yellow cap)
6. Not in use
7. Not in use
8. **Conjugate** (0.1 ml, 100x concentrated)
9. Not in use
10. Not in use
11. **Standard** (300 µl; 1 µg/ml in ethanol)
12. Not in use
13. Not in use
14. Not in use
15. Not in use
16. Not in use
17. Not in use
18. Not in use
19. Not in use

3. Pipette 100 µl of dilution buffer in duplicate (wells H1, H2, blank).
   Pipette 50 µl of dilution buffer (Bmax) in duplicate (wells A1, A2).
   Pipette 50 µl of each of the standard solutions in duplicate (wells B1,2 to G1,2 i.e. 0.05, 0.1, 0.2, 0.5, 1 and 2 ng/ml).

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

5. Pipette 25 µl of conjugate to all wells, except H1 and H2.

6. Add 25 µl of diluted stanozolol antibody to all wells, except wells H1 and H2.

7. Seal the microtiter plate and shake the plate for a few seconds on a microtiter plate shaker.

8. Incubate for 30 minutes in the dark at 20°C to 25°C.

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

10. Pipette 100 µl of substrate solution into each well.

10. Incubate 15 minutes in the dark at 20°C to 25°C.

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

12. Read the absorbance values immediately at 450 nm.

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 (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 (Preparation of reagents).

2.

Volume of standard to be pipetted (µl)	Volume of dilution buffer to be added (µl)	Obtained concentration (ng/ml)
10 µl of 1 µg/ml	990	10
200 µl of 10 ng/ml	800	2
200 µl of 10 ng/ml	1800	1
500 µl of 1 ng/ml	500	0.5
200 µl of 1 ng/ml	800	0.2
100 µl of 1 ng/ml	900	0.1
50 µl of 1 ng/ml	950	0.05

6. EQUIPMENT 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
- 4 ml glass tubes
- 15 ml tubes with screw cap (Greiner, polypropylene)
- 1 M acetic acid
- Helix pomatia juice
- sodium hydroxide
- Tert-butyl-methylether
- Nitrogen cylinder
- Distilled water
- Chloroform
- NaOH

7. PRECAUTIONS

- Stanozolol is a toxic compound. Avoid contact with mouth and skin.
- 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; 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.
- All components should be completely dissolved before use. Take special attention to the substrate, which crystallises at 4°C.
- 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 stanozolol and 16β-hydroxystanozolol into different matrixes. We have provided you information on different techniques but **other alternative methods may be used.**

8.1. Urine
Urine samples can be analysed after a 5 times dilution step in dilution buffer. A lower detection limit can be obtained using the extraction method.
Especially for dark and/or dirty urines, this extraction method is advised.

8.1.1 Direct method
- Add 800 µl of dilution buffer to 200 µl of the urine sample, mix and check pH value (pH 7 ± 0.5). Alternatively, sample extraction buffer (PBST)* can be used to dilute the sample. However, in this case, the standard range should be prepared in the sample extraction buffer.
- Centrifuge the mixture (10 minutes, 2000 x g) and use 50 µl portion of the supernatant for the test.

8.1.2 Extraction method
- Transfer 0.5 ml of urine into a centrifugal vial.
- Add a few drops of 1 M acetic acid to adjust the pH to 4.8.
- Mix and add 13 µl *Helix pomatia* juice and incubate overnight at 37°C or alternatively for 2 hours at 55°C ; cool to 20°C – 25°C.
- Add 50 µl of 5 M sodium hydroxyde and 6 ml of tert-butyl-methylether. Mix thoroughly (vortex) for 2 minutes and centrifuge (10 minutes, 2000 x g).
- Evaporate 3 ml of the upper layer (tert-butyl-methylether) at 40°C under a stream of nitrogen.
- Dissolve the residue in 500 µl of dilution buffer (0.25 ml of urine/500 µl of buffer). Use 50 µl portions for the test.

8.2. Faeces
- Weigh 1 g of homogenised sample in a centrifugal vial, add 2.5 ml of distilled water and dissolve by shaking.
- Add 150 µl of 1 N sodium hydroxyde and 4 ml of tert-butyl-methylether. Mix thoroughly (vortex) for 2 minutes, centrifuge (10 minutes, 2000 x g) and freeze the aqueous layer.
- Transfer the supernatant into a new vial and evaporate.
- Add 0.5 ml of chloroform, 2 ml of 0.1 N HCl, mix thoroughly (vortex) for 2 minutes and centrifuge (10 minutes, 2000x g).
- Transfer 1 ml of the supernatant into a new vial.
- Add 150 µl of 1N sodium hydroxyde (NaOH), 2 ml of tert-butyl-methylether and mix thoroughly (vortex) for 2 minutes, centrifuge (10 minutes, 2000 x g), freeze the aqueous layer.
- Transfer the supernatant (organic layer) into a new vial and reduce to dryness at 40°C under a weak nitrogen flow.
- Dissolve the dry residue in 1 ml of dilution buffer and vortex.
- Use 50 µl per well in the assay.

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.

Dilution buffer
The dilution buffer is delivered 10x concentrated (e.g. 1 ml of concentrated buffer + 9 ml of distilled water). Prepare dilutions freshly before use. This buffer is used for standard preparation, dilution of the concentrated conjugate and for dissolving of the antibody.

Conjugate solution (100 µl)
The conjugate is delivered 100x concentrated. Spin down the conjugate in the vial by a short centrifugation step (1 minute, 1000 x g). Add 5 µl of the concentrated conjugate solution to 495 µl dilution buffer. Per 2 x 8 wells 400 µl is required. Store unused concentrated conjugate at 2°C to 8°C.

Antibody solution
Reconstitute the vial of lyophilised antibodies with 4 ml dilution buffer, mix thoroughly and store the vial immediately after use in the dark at 2°C to 8°C. For prolonged storage store aliquots at -20°C immediately after the first use.

Standard solution
In order to avoid stability problems with the diluted standards, a vial of stanozolol standard stock solution (300 µl of 1 µg/ml) is provided with the kit.
The procedure to follow for the dilution is described on page 9.

Rinsing buffer
The rinsing buffer is delivered 20x 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
The substrate solution (ready-to-use) tends to precipitate at 4°C. Take care that this vial is at 20°C to 25°C when used (keep in the dark) and mix the content before pipetting into the wells.

* Sample extraction buffer (PBST) 1 liter

$Na_2HPO_4.2H_2O$	0.96 g
KH_2PO_4	0.17 g
NaCl	9 g
Tween 20	0.5 ml
H_2O	1000 ml

Adjust pH at 7.5