METHYLTESTOSTERONE ELISA

5081MTES[4]07.20

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

EUROPROXIMA METHYLTESTOSTERONE ELISA

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The quality management system of R-Biopharm Nederland B.V. is ISO 9001:2015 certified

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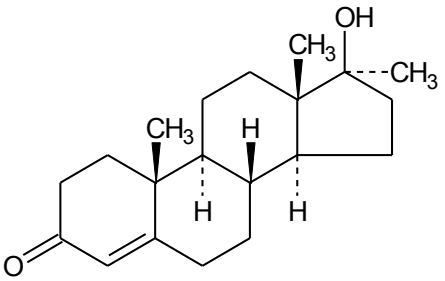
BRIEF INFORMATION

The methyltestosterone ELISA is a competitive enzyme immunoassay for measurement of the concentration of methyltestosterone. 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



Chemical structure of methyltestosterone

Methyltestosterone (17α-methyltestosterone) is a synthetic androgen used to treat men with testosterone deficiency or to treat breast cancer in women. It is commonly used by fish farmers for sex reversal in fish to obtain fast-growing all-male populations for economic gains. Furthermore, methyltestosterone is illegally used in cattle as a growth promoter.

2. PRINCIPLE OF THE METHYLTESTOSTERONE ELISA

The microtiter plate based methyltestosterone ELISA consists of one precoated plate (12 strips, 8 wells each). Antibody, Horseradish peroxidase (-HRP) labeled methyltestosterone and standard solution or sample are added to wells. Free methyltestosterone from the samples or standards and methyltestosterone-HRP conjugate compete for the specific antibody binding sites (competitive enzyme immunoassay).
After an incubation step of 1 hour the non-bound reagents are removed in a washing step. The amount of bound methyltestosterone-HRP conjugate is visualized by the addition of a substrate/chromogen solution (H_2O_2/TMB). Bound methyltestosterone-HRP conjugate transforms the colourless 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. The optical density is inversely proportional to the methyltestosterone concentration in the sample.

3. SPECIFICITY AND SENSITIVITY

The methyltestosterone ELISA utilizes antibodies raised in rabbit against protein conjugated methyltestosterone. The reactivity pattern of the antibody is:

Cross-reactivity:

Methyltestosterone	100%
Methandrolone	47%
Stanozolol	18.4%
β-Testosterone	32.7%
19-Nortestosterone	2.1%
Dihydrotestosterone	2.3%
β-Trenbolone	< 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) 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	0.042	0.1
Tissue – bovine	8.2	0.053	0.25
Tissue – fish	8.2	0.22	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:

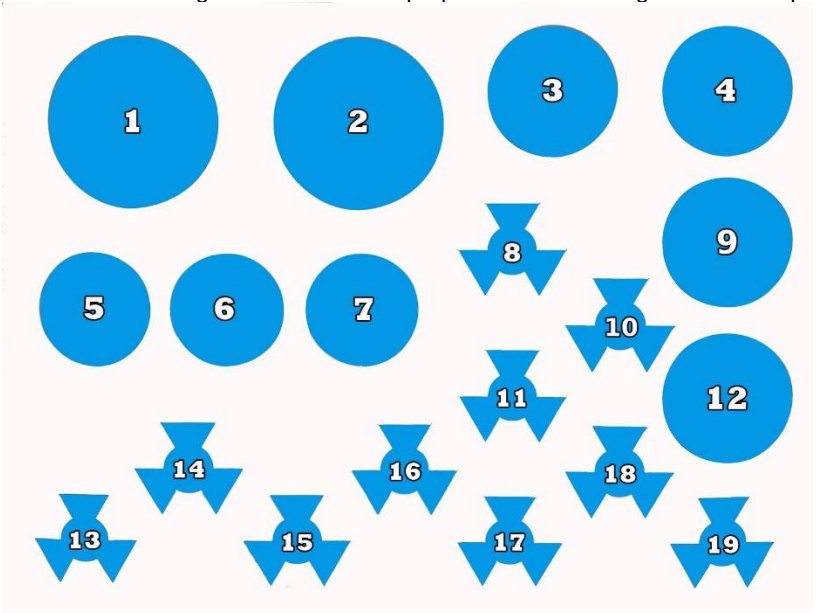
- A blue colouring of the chromogen solution before transferring it into the wells.
- A weak or no colour reaction in the zero standard wells (E450nm < 0.8).

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. D**ilution 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. Not in use
6. Not in use
7. Not in use
8. **Conjugate solution** (100 µl; 100x concentrated)
9. Not in use
10. **Antibody solution** (100 µl; 100x concentrated)
11. Not in use
12. Not in use
13. **Zero Standard solution** (2 ml, Ready-to-use)
14. **Standard solution 1** (1 ml, Ready-to-use) **0.031 ng/ml**
15. **Standard solution 2** (1 ml, Ready-to-use) **0.063 ng/ml**
16 .**Standard solution 3** (1 ml, Ready-to-use) **0.125 ng/ml**
17 .**Standard solution 4** (1 ml, Ready-to-use) **0.25 ng/ml**
18. **Standard solution 5** (1 ml, Ready-to-use) **0.5 ng/ml**
19. **Standard solution 6** (1 ml, Ready-to-use) **1.0 ng/ml**

6. EQUIPMENT AND MATERIALS REQUIRED BUT NOT PROVIDED

- Scales and weighing vessels
- Gloves
- Evaporation equipment
- 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
- Methanol 100%
- 4 ml glass tubes
- 15 ml tubes with screw cap (polypropylene)
- Distilled water
- RIDA®C18 column
- 100% methanol
- 80% (v/v) methanol in demineralized water
- 40% (v/v) methanol in demineralized water
- Beta-glucuronidase from Escherichia coli
- Potassium dihydrogen phosphate (KH_2HPO_4)
- Di-Potassium hydrogen phosphate (K_2HPO_4)

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.

Nederland shall not be liable for any damages, including special or consequential damage, or expense arising directly or indirectly from the use of this product.

8. SAMPLE PREPARATION

8.1 Urine
- Centrifuge urine: 5 min / 3000 g / 20 - 25°C (68 – 77 °F)
- Dilute 0.5 ml urine with 3 ml 75 mM potassium phosphate buffer, pH 6.8 (see chapter 9)
- Add 10 µl of β-glucuronidase
- Hydrolyze the solution for 3 h at 37°C / 98 °F (alternatively over night at room temperature 20 - 25°C (68 – 77 °F))

The hydrolyzed product is purified by means of RIDA® C18 column (flow rate: 1 drops/s)
- Rinse the column with 2 ml methanol (100%)
- Equilibrate the column with 2 ml Methanol/Tris-HCl (20/80 v/v) (see chapter 9)
- Apply sample (approx. 3.5 ml)
- Rinse the column with 2 ml Methanol /Tris-HCl (20/80 v/v)
- Rinse the column with 2 ml methanol (40%)
- Ensure that all liquid is removed from the column by pressing air or N_2 through the column
- Elute slowly (flow rate: 15 drops/min) with 1 ml methanol (80%)
- Dilute the eluate 1:2 (1+1) with demineralized water (e.g. 100 µl eluate + 100µl demineralized water)
- Use 50 µl of this solution in the ELISA test

8.2 Tissue (fish and bovine)
- Homogenise approximately 10 gram sample
- Weigh 1 gram homogenized sample into a clean tube
- Add 3 ml 100% methanol
- Mix 10 minutes, head over head (rotor)
- Centrifuge for 5 minutes at 2000 x g
- Pipette 500 µl into a glass tube, evaporate to dryness under a mild stream of nitrogen at 50˚C
- Dissolve the residue with 500 µl sample dilution buffer (see chapter 9)
- Mix thoroughly
- Use 50 µl of the sample 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 to +8ºC. Prepare reagents fresh before use.

Microtiter plate
Return unused strips into the resealable bag with desiccant and store at +2ºC to +8ºC for use in subsequent assays. Retain also the strip holder.

Dilution buffer
This buffer is used for the dilution of conjugate, antibody and samples. The dilution buffer is 10x concentrated. Before dilution (10 ml buffer + 90 ml distilled water) the concentrated buffer should be at room temperature (20ºC to 25ºC) and thoroughly mixed. Concentrated buffer can show precipitates, mix well before dilution

Sample dilution buffer
Sample dilution buffer is not provided in the kit. Prepare this buffer as follows: Take 18 ml dilution buffer, add 2 ml 100% methanol, mix and store this buffer at 4ºC until use.

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

Antibody
The antibody is 100x concentrated. Spin down the antibody in the vial by a short centrifugation step (1 min. 1000 x g). Add 5 µl of the concentrated antibody to 495 µl dilution buffer. Per 2 x 8 wells 400 µl of antibody solution is required. Store concentrated antibody immediately upon use at 2°C - 8°C

Rinsing buffer
The rinsing buffer is delivered 20x concentrated. Prepare dilutions freshly before use. For each strip 20ml of diluted rinsing buffer is used (1 ml concentrated rinsing buffer + 19 ml distilled water).

Substrate/chromogen solution
The substrate/chromogen solution (ready-to-use) tends to precipitate at +4ºC. Take care that this vial is at room temperature when used (keep in the dark) and mix the content before pipetting into the wells.

75mM potassium phosphate buffer, pH 6.8
Buffer A: dissolve 10.2 g KH_2PO_4 in 1000 ml dist. water
Buffer B: dissolve 13.06 g K_2HPO_4 in 1000 ml dist. water
Mix buffer A and buffer B (ratio 1:1) for adjustment to pH value 6.8

Methanol/Tris-HCl (20/80 v/v), pH 8.5
Dissolve 2.42 g Tris-(hydroxymethyl)-aminomethane in approx. 700 ml dist. water + 200 ml methanol (100%), adjust to pH 8.5 with 5 M HCl, fill up to 1000 ml with dist. water

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 and prepare reagents according to Chapter 9.

2. Pipette 100 µl of zero standard in duplicate (wells H1, H2, blank).
   Pipette 50 µl of zero standard (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. 0.03125, 0.0625, 0.125, 0.25, 0.5 and 1.0 ng/ml).

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

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

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

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

7. Incubate for 1 hour in the dark at room temperature (20ºC to 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 room temperature (20ºC to 25ºC).

11. Add 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.

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

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 value of absorption (logit) calculation of the standards are plotted on Y-axis versus the analyte equivalent concentration on a logarithmic X-axis.

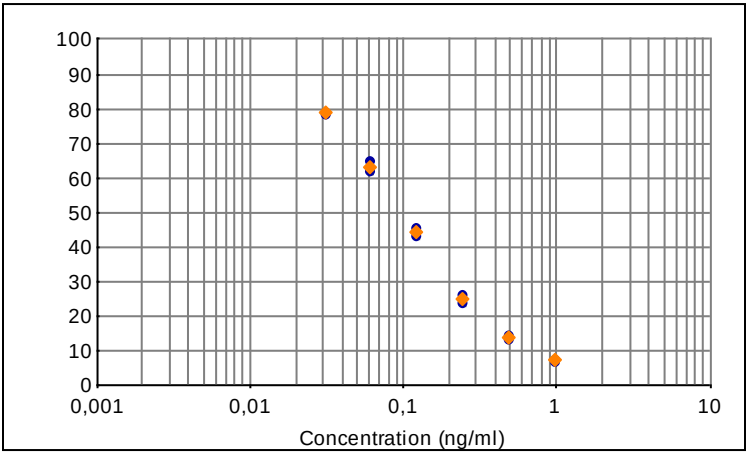


Figure 1 : Example of a calibration curve

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

Urine
To obtain the methyltestosterone content in urine samples, the calculated methyltestosterone concentration has to be multiplied by a factor 4.

Tissue
To obtain the methyltestosterone content in tissue samples, the calculated methyltestosterone concentration has to be multiplied by a factor 4.

12. LITERATURE

Amarasinghe, K., Chu, P-S., Evans, E., Reimschuessel, R., Hasbrouck, N., and Jayasuriya, H. Development of a fast screening and confirmatory method by liquid chromatography-quadrupole-time-of-flight mass spectrometry for glucuronide-conjugated methyltestosterone metabolite in tilapia. J. Agricult. Food Chem., **60**, 5084-5088, 2012.

13. ORDERING INFORMATION

For ordering the methyltestosterone ELISA kit, please use cat. code 5081MTES.

14. REVISION HISTORY

A new sample preparation for urine is presented.

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