12. LITERATURE

1. 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. LAST AMENDMENT

In this version the LOD and ccβ values for bovine tissue as well as fish tissue are listed.
Both tissue species are specifically described in the sample preparation and calculation.

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METHYLTESTOSTERONE ELISA

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

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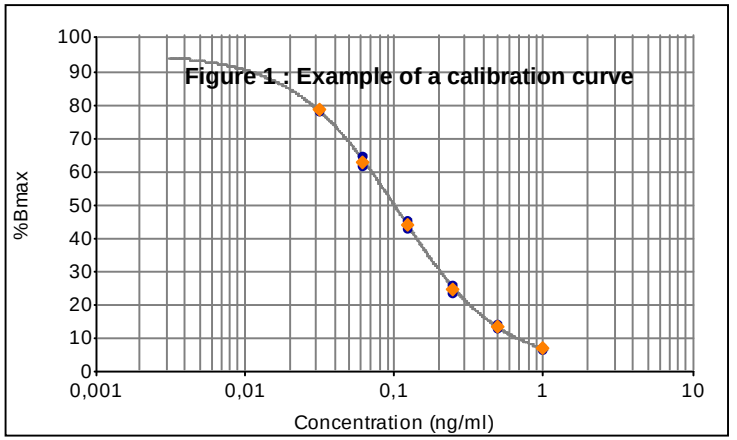


The amount of methyltestosterone in the samples is expressed as methyltestosterone equivalents. The methyltestosterone equivalents in the samples (ng/ml) corresponding to the % maximal absorbance of each extract can be read from the calibration curve.

Urine

To obtain the methyltestosterone content in urine samples, the calculated methyltestosterone concentration has to be multiplied by a factor 40.

Tissue

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

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.

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

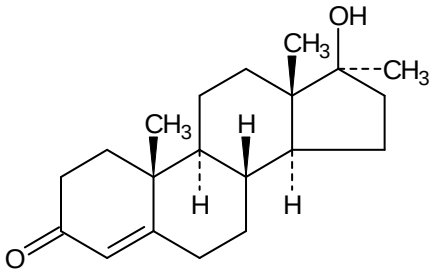
Calibration curve:
The values (% maximal absorbance) calculated for the standards are plotted on the Y-axis versus the analyte equivalent concentration (ng/ml) on a logarithmic X-axis.

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.

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 [1]. 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 Limit of detection (LOD) is calculated as: Xn+3SD and is determined under optimal conditions.

Matrix	Procedure	LOD (ppb)	ccβ (ppb)
Urine	Dilution	2.0	-
Tissue – bovine	Extraction	0.053	0.25
Tissue – fish	Extraction	0.22	0.5

4. HANDLING AND STORAGE

- Store the kit at +2ºC to +8ºC in a dark place. For repeated use store 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.
- The substrate chromogen solution can be stored in a refrigerator (+2ºC to + 8ºC) until the expiry date stated on the label.
- Any direct action of light on the substrate 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).

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. 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.

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 (10x concentrated)
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. The diluted buffer can be stored at +2℃ to +8℃.

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℃ - 8℃

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℃ - 8℃

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).

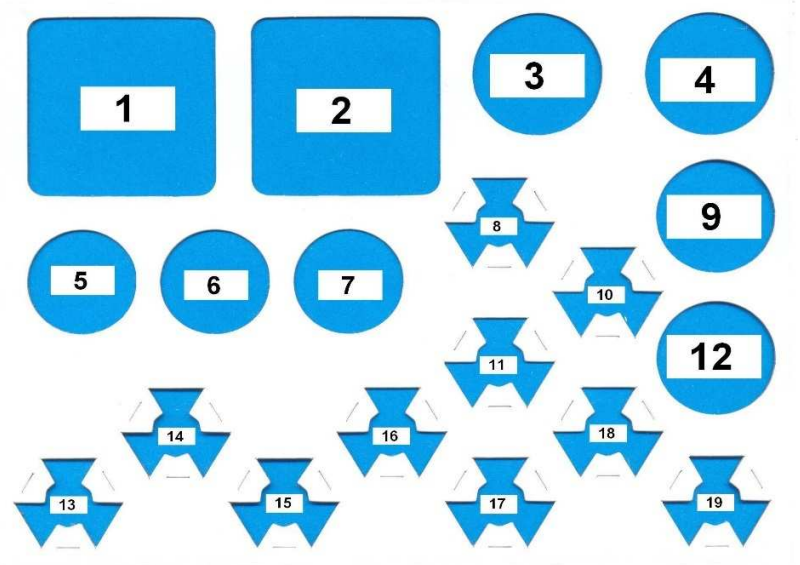
Substrate/chromogen solution
The substrate/chromogen solution (ready-to-use) tends to precipitate at +4ºC. Take care that this vial is at room temperature when used (keep in the dark) and mix the content before pipetting into the wells.

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. Not in use
11. **Antibody solution** (100 µl; 100x concentrated)
12. Not in use
13. **Zero Standard** (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 (Greiner, polypropylene)
- Distilled water

7. PRECAUTIONS

- Methyltestosterone is a toxic compound. Avoid contact with mouth and skin. Be aware that methyltestosterone 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 PREPARATION

Urine
- Homogenise sample
- Pipette 25 µl into a clean tube
- Add 975 µl of sample dilution buffer*
- Mix thoroughly
- Use 50 µl of the diluted sample in the ELISA

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*
- Mix thoroughly
- Use 50 µl of the sample in the ELISA

* see chapter 9