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Instructions for use

Testosterone rat/ mouse ELISA

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INTRODUCTION

INTENDED USE

The Testosterone rat/ mouse ELISA is a competitive immunoassay for the quantitative measurement of testosterone in rat and mouse serum or plasma. For research use only.

SUMMARY AND EXPLANATION

Testosterone is a steroid hormone from the androgen group synthesized by the Leydig cells in the testes in males, the ovaries in females, and adrenal glands in both sexes. It exerts a wide-ranging influence over sexual behaviour, muscle mass and strength, energy, cardiovascular health and bone integrity.
Testosterone biosynthesis coincides with the spermatogenesis and fetal Leydig cell differentiation in the male rat. Several in vivo models including hormone-suppression, hormone-restoration and hypophysectomy were established for the study of the hormonal regulation of spermatogenesis by testosterone (1-3).
In the Brown Norway rat, serum testosterone levels decrease with aging, accompanied by increases in serum FSH. The capacity of Leydig cells to produce testosterone is higher in young than in old rats (4). Testosterone secreted during late gestational and neonatal periods causes significant brain sexual dimorphism in the rat. This results in both sex-specific behaviour and endocrinology in adults (5).
Analyses concerning the regulation of synthesis reveal that testosterone is able to regulate its own synthesis and indicate that this autoregulation is the result of rapid, specific inhibition by testosterone of 17 alpha-hydroxylase activity (6).

PRINCIPLE

The Testosterone rat/ mouse ELISA Kit is a solid phase enzyme-linked immunosorbent assay (ELISA), based on the principle of competitive binding. An unknown amount of testosterone present in the sample and a defined amount of testosterone conjugated to horseradish peroxidase compete for the binding sites of testosterone antiserum coated to the wells of a microplate. After one-hour incubation on a shaker the microplate is washed four times. After addition of the substrate solution the concentration of testosterone is inversely proportional to the optical density measured.

WARNINGS AND PRECAUTIONS

1. For professional use only.
2. Before starting the assay, read the instructions completely and carefully. Use the valid version of the package insert provided with the kit. Be sure that everything is understood.
3. Do not mix reagents of different lots. Do not use expired reagents.
4. The microplate contains snap-off strips. Unused wells must be stored at 2 – 8 °C in the sealed foil pouch and used in the frame provided.
5. Avoid contact with Stop Solution. It may cause skin irritation and burns.
6. Pipetting of samples and reagents must be performed as quickly as possible and in the same sequence for each step.
7. Change pipette tips between samples and reagents to avoid carry over contamination.
8. Use reservoirs only for single reagents. This especially applies to the substrate reservoirs. Using a reservoir for dispensing a substrate solution that had previously been used for the conjugate solution may turn solution colored. Do not pour reagents back into vials as reagent contamination may occur.
9. Mix the contents of the microplate wells thoroughly to ensure good test results. Do not reuse microwells.
10. Do not let wells dry during assay; add reagents immediately after completing the rinsing steps.

REAGENTS

Reagents provided

AR E-8031 96 Microtiterplate, 12 x 8 (break apart) strips with 96 wells;
Wells coated with anti-testosterone antibody.

Calibrators - Ready To Use.
Listed below are approximate concentrations, please refer to vial labels for exact concentrations:

Cat. no.	Symbol	Calibrator	Concentration	Volume/ Vial
AR E-8001	STANDARD A	Calibrator 0	0 ng/ml	0.3 ml
AR E-8002	STANDARD B	Calibrator 1	0.1 ng/ml	0.3 ml
AR E-8003	STANDARD C	Calibrator 2	0.4 ng/ml	0.3 ml
AR E-8004	STANDARD D	Calibrator 3	1.5 ng/ml	0.3 ml
AR E-8005	STANDARD E	Calibrator 4	6 ng/ml	0.3 ml
AR E-8006	STANDARD F	Calibrator 5	25 ng/ml	0.3 ml

AR E-8013 INC-BUFF Incubation Buffer, 1 vial 11 ml, ready to use;

AR E-8040 CONJUGATE Enzyme Conjugate, 1 vial, 7 ml, ready to use;
Testosterone conjugated to horseradish peroxidise.

AR E-0055 SUBSTRATE Substrate Solution, 1 vial, 22 ml each, ready to use;
contains tetramethylbenzidine (TMB) and hydrogen peroxide in a buffered matrix.

AR E-0080 STOP-SOLN Stop Solution, 1 vial, 7 ml, ready to use;
contains 2 N Hydrochloric Acid solution.

AR E-0030 WASH-CONC 10x Wash Solution, 1 vial, 50 ml (10X concentrated);
see „Preparation of Reagents“.

Note: Additional Calibrator 0 for sample dilution is available upon request.

Materials required but not provided

- Centrifuge
- A microtiter plate reader capable for endpoint measurement at 450 nm
- Microplate mixer operating more than 600 rpm
- Vortex mixer
- Calibrated variable precision micropipettes (10 µl, 50 µl, 100 µl, 200 µl).
- Absorbent paper
- Distilled or deionized water
- Timer
- Semi logarithmic graph paper or software for data reduction

Reagent preparation

All reagents should be at room temperature before use.

Wash Solution:
Dilute 50 ml of 10X concentrated Wash Solution with 450 ml deionized water to a final volume of 500 ml.
The diluted Wash Solution is stable for at least 3 months at room temperature.

ASSAY PROCEDURE

General Remarks

- All reagents and specimens must be allowed to come to room temperature before use. All reagents must be mixed without foaming.
- Once the test has been started, all steps should be completed without interruption.
- Use new disposal plastic pipette tips for each standard and sample in order to avoid cross contamination.
- Absorbance is a function of the incubation time and temperature. Before starting the assay, it is recommended that all reagents are ready, caps removed, all needed wells secured in holder, etc. This will ensure equal elapsed time for each pipetting step without interruption.
- As a general rule the enzymatic reaction is linearly proportional to time and temperature.

ASSAY PROCEDURE

Each run must include a standard curve.

1. Prepare a sufficient number of microplate wells to accommodate calibrators and samples in duplicates.
2. Dispense 10 µl of each Calibrator and Sample with new disposable tips into appropriate wells.
3. Dispense 100 µl of Incubation Buffer into each well.
4. Add 50 µl Enzyme Conjugate into each well.
5. Incubate for 60 minutes at room temperature on a Microplate mixer.
   Important Note:
   Optimal reaction in this assay is markedly dependent on shaking of the microplate!
6. Discard the content of the wells and rinse the wells 4 times with diluted Wash Solution (300 µl per well). Remove as much Wash Solution as possible by beating the microplate on absorbent paper.
7. Add 200 µl of Substrate Solution to each well.
8. Incubate without shaking for 30 minutes in the dark.
9. Stop the reaction by adding 50 µl of Stop Solution to each well.
10. Determine the absorbance of each well at 450 nm. It is recommended to read the wells within 15 minutes.

CALCULATION OF RESULTS

1. Calculate the average absorbance values for each set of calibrators, controls and patient samples.
2. Using semi logarithmic graph paper, construct a standard curve by plotting the mean absorbance obtained from each standard against its concentration with absorbance value on the vertical (Y) axis and concentration on the horizontal (X) axis.
3. Using the mean absorbance value for each sample, determine the corresponding concentration from the calibration curve.
4. Automated method: Computer programs using cubic spline, 4 PL (4 Parameter Logistics) or Logit-Log are recommended.
5. The concentration of the samples can be determined directly from this calibrator curve. Samples with concentrations higher than that of the highest calibrator have to be further diluted. For the calculation of the concentrations, this dilution factor has to be taken into account.

Conversion to SI units:

Example of Typical Calibrator Curve

Following data are intended for illustration only and should not be used to calculate results from another run.

Standard	Absorbance Units
Calibrator 0 (0 ng/ml)	2.478
Calibrator 1 (0.1 ng/ml)	2.078
Calibrator 2 (0.4 ng/ml)	1.668
Calibrator 3 (1.5 ng/ml)	1.170
Calibrator 4 (6.0 ng/ml)	0.645
Calibrator 5 (25.0 ng/ml)	0.330

EXPECTED NORMAL VALUES

In order to determine the normal range of serum testosterone in rat, samples from 35 male rats and 20 female rats were collected and analyzed using the Testosterone rat/mouse ELISA kit. The following ranges are calculated with the results of this study.

	Range (ng/ ml)	Mean (ng/ ml)
Male ♂	0.66 – 5.4	3.06
Female ♀	0.11 – 0.31	0.21

In further studies serum samples of 10 mice were collected between 11.00 am and 3.00 pm und analyzed in a similar manner.

	Range (ng/ ml)	Mean (ng/ ml)
Male mice ♂	1.7 – 14.4	6.78

It is recommended that each laboratory establish its own normal range since testosterone levels can vary due to handling and sampling techniques.

PERFORMANCE CHARACTERISTICS

ANALYTICAL SENSITIVITY

The lowest analytical detectable level of testosterone that can be distinguished from the Zero Calibrator is 0.066 ng/ml at the 2SD confidence limit.

SPECIFICITY

The following materials have been evaluated for cross reactivity. The percentage indicates cross reactivity at 50% displacement compared to Testosterone.

Steroid	% Cross reaction
Dihydrotestoterone	69.6
Androstenedione	< 0.1
Androsterone	< 0.1
Epiandrosterone	< 0.1
Dihydroandrosterone	< 0.1
Dihydroxyandrosterone	7.4
Estron	< 0.1
Estradiol	< 0.1
Estriol	< 0.1
Cortisol	< 0.1
11-Deoxycortisol	< 0.1
Progesterone	< 0.1
17OH-Progesterone	< 0.1

REPRODUCIBILITY

Intra-Assay

The intra-assay variation was determined by 20 replicate measurements of 3 serum samples within one run. The within-assay variability is shown below:

Mean (ng/ml)	3.23	1.44	0.84
SD	0.21	0.12	0.09
CV (%)	6.50	8.06	11.07
n =	20	20	20

Inter-Assay

The inter-assay (between-run) variation was determined by duplicate measurements of 3 serum samples over 10 days.

Mean (ng/ml)	0.29	1.23	9.50
SD	0.03	0.11	0.88
CV (%)	11.3	9.3	9.3

RECOVERY

Using the Testosterone rat/mouse Calibrator Matrix three spiking solutions were prepared (A = 50 ng/ml, B = 100 ng/ml, C = 150 ng/ml). A 25 µl aliquot of each solution was spiked into 475 ml of six different rat sera with low testosterone concentrations for a spiking ratio of 1 to 20, leaving the serum matrix of the spiked samples relatively intact. All samples were then measured by Testosterone rat/mouse procedure. To calculate expected values 95% of the unspiked values were added to 5% of the spiking solution concentrations (2.5, 5 and 7.5 ng/ml, respectively).

Serum	Spiking Solution	Observed (O)	Expected (E)	O/E %
1	-	0,31	-	-
	A	3,15	2,81	112%
	B	5,11	5,31	96%
	C	7,27	7,18	93%
2	-	0,40	-	-
	A	3,42	2,90	118%
	B	5,88	5,40	109%
	C	7,90	7,90	100%
3	-	0,36	-	-
	A	2,88	2,86	96%
	B	5,50	5,36	89%
	C	7,50	7,86	91%
4	-	0,25	-	-
	A	2,65	2,75	96%
	B	4,65	5,25	89%
	C	7,08	7,75	91%
5	-	0,38	-	-
	A	3,17	2,88	110%
	B	4,79	5,38	89%
	C	7,36	7,88	93%
6	-	0,28	-	-
	A	2,78	2,78	100%
	B	4,61	5,38	86%
	C	7,22	7,78	93%

LINEARITY

Five native serum samples were assayed undiluted and diluted with the calibrator matrix.

Serum	Dilution	Observed (O)	Expected (E)	O/E %
3	native	2.54	-	-
	1 in 2	1.22	1.27	96%
	1 in 4	0.61	0.64	95%
	1 in 8	0.34	0.32	106%
15	native	1.85	-	-
	1 in 2	0.86	0.93	92%
	1 in 4	0.43	0.46	93%
	1 in 8	0.21	0.23	91%

LIMITATIONS OF PROCEDURE

Reliable and reproducible results will be obtained when the assay procedure is performed with a complete understanding of the package insert instruction and with adherence to good laboratory practice. Any improper handling of samples or modification of this test might influence the results.

DRUG INTERFERENCES

Until now no substances (drugs) are known influencing the measurement of rat or mouse testosterone in serum and plasma. Lipemic and haemolysed samples can cause false results.

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