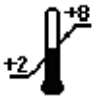
BIOGENIX
INC. PVT. LTD.

Testosterone ELISA

Enzyme Immunoassay for Quantitative Determination of Testosterone in Human Serum

Instructions for use

1. SYMBOL LEGEND

IVD	In vitro diagnostic medical device	CE	EC Declaration of conformity
REF	Catalogue number	LOT	Batch code
	Use by		Manufacturer
	Date of manufacture		Temperature limitation
	Contains sufficient for <n> tests		Consult operating instructions
MP	Coated microplate (96 wells)		Biological risks
WASH P 20X	Wash solution, 20X concentrated	CONJ	Conjugate
CAL	Calibrators	SUB	Substrate
CONTROL	Control	STOP	Stop solution
RCNS	Reconstitute with specified volume of liquid	OD	Optical density
 Warning	Irritant	H2O	Deionized or distilled water
		TRIAL 5000X	Trial

2. INTENDED USE

Testosterone kit is provided for the **quantitative** determination of **testosterone** in human serum.

Testosterone is a steroid hormone with a molecular mass of 288.4 Da. Testosterone is synthesized mainly in testicles and, in sufficiently less extent, in ovaries and adrenal cortex. The determination of serum testosterone is a valuable tool for investigation of testis function and diagnostics of some adrenal, ovary and testicle tumors as well as female hirsutism.

3. PRINCIPLE OF THE TEST

Testosterone kit is a competitive solid phase enzyme immunoassay. During the incubation testosterone of tested samples and horseradish peroxidase (HRP) labeled testosterone bind to the antibodies coated onto the inner surface of the microplate wells until balance between them occurs. Separation of free and bound to antibodies testosterone and conjugate testosterone-peroxidase occurs while extracting the contents of the wells. The amount of bound conjugate is inversely proportional to the quantity of testosterone in the sample (fig. 1).

During the incubation with TMB solution the colour is developing. The intensity of the colour is inversely proportional to the testosterone concentration in specimens. The testosterone concentration in the patient sample is read from a standard curve that is processed in each assay.

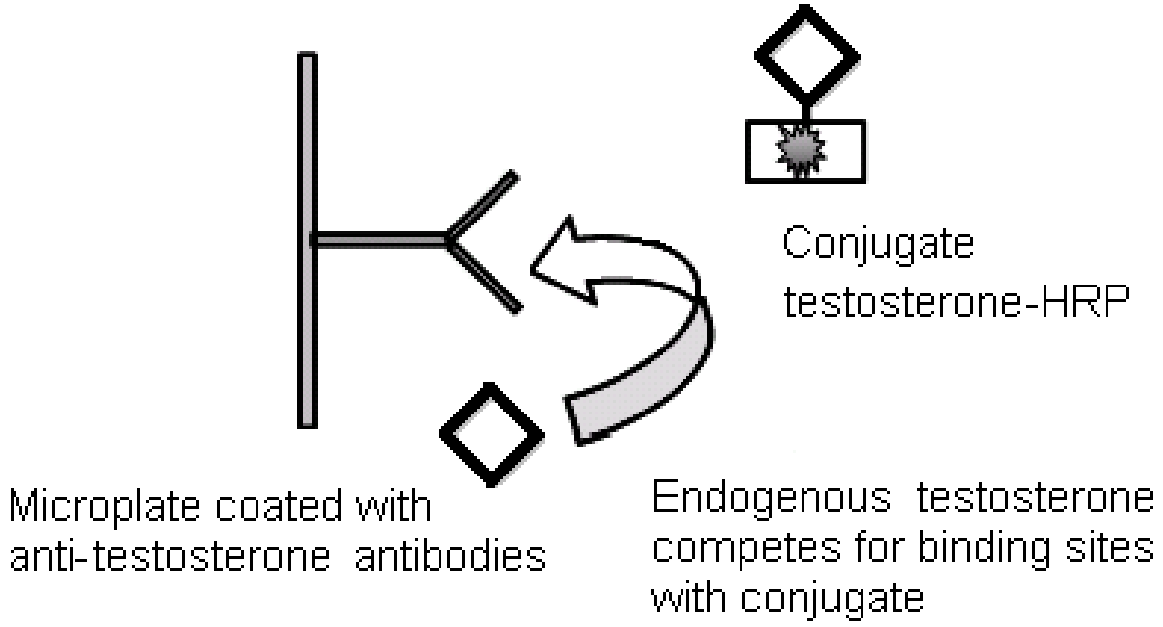


Fig. 1. Assay scheme

4. STORAGE AND STABILITY OF THE KIT

Expiry date of the kit is printed on the box label; expiration date for each component is printed on the respective label.

Testosterone kit should be stored at +2…+8 °C upon receipt, preferably in the original kit box, until expiration date. Storage at +25 °C is allowed but for no more than 5 days.

Shelf life of the kit is 18 months.

After initial opening the kit is stable until expiration date if stored at +2…+8 °C.

If used in several separate experiments, after initial opening kit contents should be stored as follows:

- unused strips: in a firmly closed resealable zipper bag at +2…+8 °C until expiration date;
- vials with calibrators, control (liquid) and conjugate: at +2…+8 °C until expiration date; vials with calibrators and control (reconstituted): at +2…+8 °C for no more than 1 month after opening;

- vial with substrate: at +2…+8 °C until expiration date, protected from light;
- vials with concentrated Trial, concentrated wash solution and stop solution: at +2…+8 °C until expiration date;
- wash solution and Trial solution prepared for use: at room temperature (+18…+25 °C) for no more than 5 days or at +2…+8°C for no more 4 weeks, in a firmly closed bottle.

Damaged Test Kits

In case of any severe damage of the test kit or components, it has to be informed in writing, during one week after receiving the kit. Usage of severely damaged single components for a test run is not recommended.

5. SAMPLE COLLECTION AND STORAGE

Collect blood by venipuncture in a tube without anticoagulants. Allow blood to clot. Centrifuge the specimens to separate serum from the blood corpuscles.

Do not use plasma, haemolyzed (bright red) or lipemic (milky) serum samples as well as samples containing sodium azide as preservative.

Store serum samples at +2…+8 °C for no more than 5 days. Aliquot and freeze samples for longer storage (–20 °C and lower). Avoid repeated freezing.

6. EXPECTED VALUES

Serum samples collected between 9 and 11 a.m. from 70 apparently healthy people, both males and females, between the ages of 21–45, were assayed with **Testosterone kit**. The results are listed below. These limits should be considered as guidelines only.

Group	Number of samples	Mean (nmol/L)	Range (nmol/L)
Female	37	1.8	0.5-4.3
Male	33	19.2	12.1-38.3

It is highly recommended each laboratory to determine its own reference range of testosterone concentrations.

7. QUALITY CONTROL

It is recommended to use control samples according to the state and federal regulations. The use of control samples is advised to assure the day to day validity of results.

8. REAGENT PREPARATION

Allow all the reagents to reach room temperature (+18…+25 °C), and then thoroughly stir.

MP Keep **microplate** at room temperature for at least 30 minutes before opening the bag. Place required number of strips onto strip holder. Place unused strips into the resealable zipper bag and reseal duly.

CAL **CONTROL** **Calibrators and Control**

Liquid calibrators and control are ready to use.

Prepare lyophilized calibrators and control as follows. Gently tap on the vial caps to knock off all the dry matter. Open the vials and carefully place the caps upside down on the clean dry surface. Add 0.5 mL of distilled or deionized water to each vial with lyophilized calibrators and control, close vials with the corresponding caps and leave for 10 min at room temperature without stirring. Then stir gently avoiding foaming, until the dry matter is completely dissolved. Leave for another 10 minutes at room temperature stirring gently periodically. Make sure that no dry matter is left on the caps and walls of the vials.

WASH P Prepare required volume of **wash solution** by dilution of the concentrate 20-fold with distilled or deionized water. For example:

5 mL of **WASH P** **20X** + 95 mL of water.

Mix thoroughly, avoid foaming.

SUB Protect **substrate** from direct light.

9. SAMLE PREPARATION

Allow samples to reach room temperature. Stir samples gently in order to ensure homogeneity.

10. PERFORMANCE CHARACTERISTICS OF THE ASSAY

10.1. Calibration-Traceability

Testosterone kit was calibrated against the Working Standard, which had been manufactured by gravimetric method based on weighing purified synthetic testosterone into analyte-free matrix.

10.2. Specificity

Cross-reaction of anti-testosterone monoclonal antibodies with different steroids is shown below.

Steroid	Cross-reaction, %
Testosterone	100.0
5-α-Dihydrotestosterone	9.8
Androstendiol	2.0
Androstenolone	0.2
Androstandione	1.3
Dehydroepiandrosterone	0.2
Cortisol	0.04
Progesterone	0.1
Estrone	0.06
Estradiol	0.9
Estriol	0.02

10.3. Analytical Sensitivity

Analytical sensitivity of **Testosterone kit,** i.e. concentration, that can be distinguished from zero calibrator is 0.2 nmol/L. It was defined as mean OD of 10 replicates of calibrator 0 minus 2 SD (for manual and Alisei kits).

10.4. Measurement Range

Testosterone kit was validated for measurement of testosterone concentration within the concentration diapason of 0.2 - 50 nmol/L(for manual test) and 0.2 - 100 nmol/L (for “Alisei”).

10.5. Measurement Units

In **Testosterone kit** the concentrations of calibrators are specified in nmol/L. To convert into ng/mL, multiply the concentration in nmol/L by 0.288.

10.6. Intra- and Inter-Assay Variation (Precision)

For **intra-assay CV** determination, 8 serum samples were run, each in 9 replicates. The results are shown below.

Manual kit

Sample	**Mean testosterone concentration, nmol/L**	**Intra-assay CV**	
		SD	**CV, %**
HS 1	1.14	0.177	15.4
HS 2	1.37	0.209	15.3
HS 3	4.68	0.295	6.3
HS 4	13.0	0.75	5.8
HS 5	16.9	0.42	2.5
HS 6	17.2	0.60	3.5
HS 7	19.6	0.59	3.0
HS 8	26.2	0.74	2.8

HS 7	19.11	0.488	2.6
HS 8	29.56	0.726	2.5

For **inter-assay CV** determination, 10 serum samples were assayed 4 times by different operators with 1-week interval. Each specimen was run in 2 replicates. The results are shown below.

Sample	**Mean testosterone concentration, nmol/L**				**Inter-assay CV**	
	Assay 1	**Assay 2**	**Assay 3**	**Assay 4**	**SD**	**CV, %**
HS 1	0.71	0.76	0.79	0.69	0.046	6.2
HS 2	1.34	1.44	0.97	1.07	0.221	18.4
HS 3	1.34	1.33	1.50	1.29	0.093	6.8
HS 4	2.07	1.93	1.93	1.95	0.067	3.4
HS 5	2.33	2.60	2.14	2.25	0.196	8.4
HS 6	12.3	14.2	14.1	12.9	0.92	6.9
HS 7	12.7	14.7	14.1	13.9	0.84	6.0
HS 8	13.8	15.1	17.7	15.6	1.60	10.3
HS 9	14.3	17.0	16.4	15.3	1.20	7.6
HS 10	16.4	18.4	16.6	16.5	0.96	5.7

11. LIMITATION OF THE METHOD

Any clinical diagnosis should not be based on the results of in vitro diagnostic methods alone. For diagnosis establishment, a physician is supposed to consider all available clinical and laboratory findings.

12. SAFETY PRECAUTIONS

- **This kit is for in vitro diagnostic use only.** Operator should follow the manual closely in order to ensure reliable data. The manual is valid for the present kit only, within the listed composition. Any substitution of kit components is not allowed by CE regulations.

- Do not use kit or its components after expiry date indicated on the label. Take into account stability period for reconstituted reagents.

- Do not mix or use together reagents from different lots of the kit except substrate, stop solution and wash solution.

- Do not use substrate, stop solution and wash solution supplied by other vendors.

- Use only “P”-labeled wash solution.

- Note that stop solution is 1 N HCl solution. Avoid contacts with skin and mucosa. In case of contact rinse affected area thoroughly with plenty of water and seek medical advice.

- Take into account the following common procedural notes:

  - always pipette reagents into wells immediately after washing procedure;
  - avoid contamination of the solutions;
  - in case of partial use of the kit, dispense only required volume of the reagent into the tray;
  - do not pour unused reagents back into the original vials;
  - avoid exposure to direct sunlight during incubations;

- always pipette reagents in the same order to minimize reaction time differences between wells; the total dispensing time for the calibrators, control and samples must not exceed 15 min;

- the incubation temperature for all the immunological reactions must be kept at recommended by this instruction levels;

- do not touch the bottom of the wells;

- calibrators should be measured in each separate assay. It is also recommended to measure each time testosterone concentration in the control.

- TMB solution should be colourless. Light colouring of solution is admissible. Avoid direct exposure of substrate to sunlight.

-  Source materials of human origin used for kit components preparation were tested and found negative for HBsAg, anti-HIV and anti-HCV antibodies. However, none of known laboratory test guarantees absence of these viral agents. Therefore, all kit components and patient's samples should be handled as potentially hazardous.

-  After usage strips, calibrators, control, specimens and all consumables which contacted with specimens during handling, storage or assay (tubes, vials, gloves, pipette tips etc.) should be collected separately and sterilized by autoclaving. Instead of autoclaving pipette tips may be sterilized by disinfectant treatment. After sterilization all components and expendable materials may be utilized as non-dangerous garbage. Other components of the kit should be discarded into conventional garbage.

- During manual washing procedure do not discard the contents of the wells directly to drainage. Use a container with disinfectant solution.

- As the kit contains potentially hazardous material, the following precautions should be taken:
  - do not smoke, eat or drink while performing the assay;
  - always use protective gloves;
  - never pipette material by mouth;
  - in case of spilling, wipe up the spills promptly and wash affected area thoroughly using decontaminant.
- GLP including all general and individual regulations should be applied for the kit usage.


As the kit contains irritant (CONJ, CAL, CONTROL), the following precautions should be observed:

- P261 - Avoid breathing spray;
- P272 - Contaminated work clothing should not be allowed out of the workplace;
- P280 - Wear protective gloves/protective clothing/eye protection;
- P302+P352 - IF ON SKIN: Wash with plenty of soap and water;
- P333+P313 - If skin irritation or rash occurs: Get medical advice/attention;
- P363 - Wash contaminated clothing before reuse;
- P501 - Dispose of contents/container in accordance with national regulation.

Precautionary statements according to Regulation EC № 1272/2008.

13. MATERIAL PROVIDED

13.1. Material Provided

MP	**Microplate:** 12 breakable 8-well strips (total 96 wells) coated with anti-testosterone monoclonal antibodies	1 pcs
CONJ	**Conjugate:** solution containing testosterone conjugated with HRP	18 mL, ready to use
0–5 CAL	**Testosterone calibrators:** protein-based solutions or lyophilized preparations containing known testosterone concentrations – 0; 0.5; 1.5; 5; 15; 50 nmol/L. The concentrations of calibrators may be different for schemes with or without shaking. For exact testosterone concentrations, see vial labels.	**6 vials,** 0.5 mL each; ready to use or lyophilized preparations
CONTROL	**Testosterone control:** protein-based solution or lyophilized preparation containing known testosterone concentration. The range of testosterone concentration may be different for schemes with or without shaking. For exact range of testosterone concentration see vial label.	0.5 mL, ready to use or lyophilized preparation
SUB	**Substrate (TMB solution):** 3,3',5,5'-tetramethylbenzidine solution in citrate buffer containing hydrogen peroxide	14 mL, ready to use
WASH P 20X	**Wash solution P, 20X concentrated**: surfactant in buffered saline, sufficient for preparation of 560 mL of solution	2x14 mL, concentrated
STOP	**Stop solution:** 1 N HCl solution	14 mL, ready to use

13.2. Equipment and Materials Required but not provided

- 1-channel calibrated variable precision pipettes, with disposable tips;
- 8-channel calibrated variable precision pipette, with disposable tips;
- microplate incubator (+37 °C and room temperature (+18…+25 °C) or microplate incubator/shaker (+37 °C and room temperature (+18…+25 °C), shaking speed 500–800 rpm);
- manual or automatic equipment for rinsing wells;
- calibrated microplate reader (450 nm);
- vortex tube mixer;
- deionized or distilled water;
- graduated beaker and cylinder of appropriate volume;
- latex or plastic gloves;
- trays for pipetting reagents with 8-channel pipette;
- disinfectant;
- absorbent material (for manual wash).

13.3. Test Procedure

Testosterone kit is designed for 96 tests. This is sufficient for the **quantitative assay** of 40 unknowns, 6 calibrators, 1 control and 1 blank (OD of TMB solution) in duplicates, provided that all the strips are used simultaneously.

13.3.1. Assay Procedure

13.3.1.1. Protocol with shaking

(See assay scheme, section 13.5)

A. Pipette**:**

- **50 μL** of **calibrators** **CAL** (0-5),

control **CONTROL** and patient's samples in duplicates into the respective wells**;**

Leave wells A1-A2 empty for blank!

B. Pipette

- **150 μL** of **conjugate** **CONJ** into each well, **except wells A1-A2**.

Note: total time of dispensing must not exceed 15 minutes, otherwise the test result may be unreliable, because the time of incubation will substantially vary for different samples.

C. Incubate strips for **90 minutes while shaking (500–800 rpm) at room temperature (+18…+25 ºC)**.

D. Wash 4 times, as described in section 13.3.2.

E. Pipette **100 μL** of **substrate** **SUB** into each well (including blank); incubate **at room temperature in the dark for 15-30 minutes**, depending on the colour intensity, or **10 minutes while shaking (500-800 rpm) at +37 ºC**.

F. Pipette **100 μL** of **stop solution** **STOP** into each well (including blank) in the same sequence and at the same speed as used for dispensing TMB substrate. Shake for 1–2 min at room temperature.

G. Read OD at **450 nm within 20 min**.

13.3.1.2.

Protocol without shaking

(See assay scheme, section 13.6)

A. Pipette**:**

- **50 µL** of **calibrators** **CAL** (0-5),

control **CONTROL** and patient's samples in duplicates into the respective wells**;**

Leave wells A1-A2 empty for blank!

B. Pipette

- **150 µL** of **conjugate** **CONJ** into each well, **except wells A1-A2**.

Note: total time of dispensing must not exceed 15 minutes, otherwise the test result may be unreliable, because the time of incubation will substantially vary for different samples.

C. Incubate strips for **90 minutes at room temperature (+18…+25 ºC)** (pre-shake for 1-2 minutes at room temperature).

D. Wash 4 times, as described in section 13.3.2.

E. Pipette **100 µL** of **substrate** **SUB** into each well (including blank); incubate **at room temperature in the dark for 15-30 minutes**, depending on the colour intensity.

F. Pipette **100 µL** of **stop solution** **STOP** into each well (including blank) in the same sequence and at the same speed as used for dispensing TMB substrate. Shake for 1–2 min at room temperature.

G. Read OD at **450 nm within 20 min**.

13.3.2. Wash Procedure

It is advisable to use an automatic microplate washer set at 4 wash cycles and a volume of 300 µL of wash solution per well per cycle.

If an automatic washer is not available, the wash procedure can be carried out manually as follows:

- remove the contents of the wells into a container with disinfectant;
- dispense 300 µL of wash solution (prepared according to section 8) into each well, shake the plate carefully for 5–10 sec and remove the contents of the wells; repeat 4 times;
- strike the wells sharply on absorbent material to remove any liquid residue.

13.4. Data Processing

If the reader cannot be adjusted to zero using the substrate blank in wells A1-A2, subtract mean OD value of wells A1-A2 from all OD values before further calculations.

Example:

OD (Cal 0) measured = 2.28 and OD (blank) = 0.06;

OD (Cal 0) calculated = 2.28 – 0.06 = 2.22

13.4.1. Data Reliability (for OD Measured at 450 nm)

The data should meet the following criteria:

- average blank OD (in wells A1-A2) ≤0.100;
- average OD of Cal 0 ≥1.0 (after blank subtraction);
- control’s concentration must fall within the acceptability range that is shown on the vial label.

If the data obtained do not meet the criteria, the results are considered unreliable and the test should be repeated.

13.4.2. Quantitative Determination

Specialized software for quantitative determination is recommended. Mean OD of calibrators are plotted versus their respective testosterone concentrations using 4PL or 5PL fit (see typical standard curve, fig. 2). Calculate concentration of testosterone in samples using standard curve.

Any extrapolation of the standard curve to testosterone concentration above the nominal value of the calibrator 5 (approximately 50 nmol/L) is forbidden.

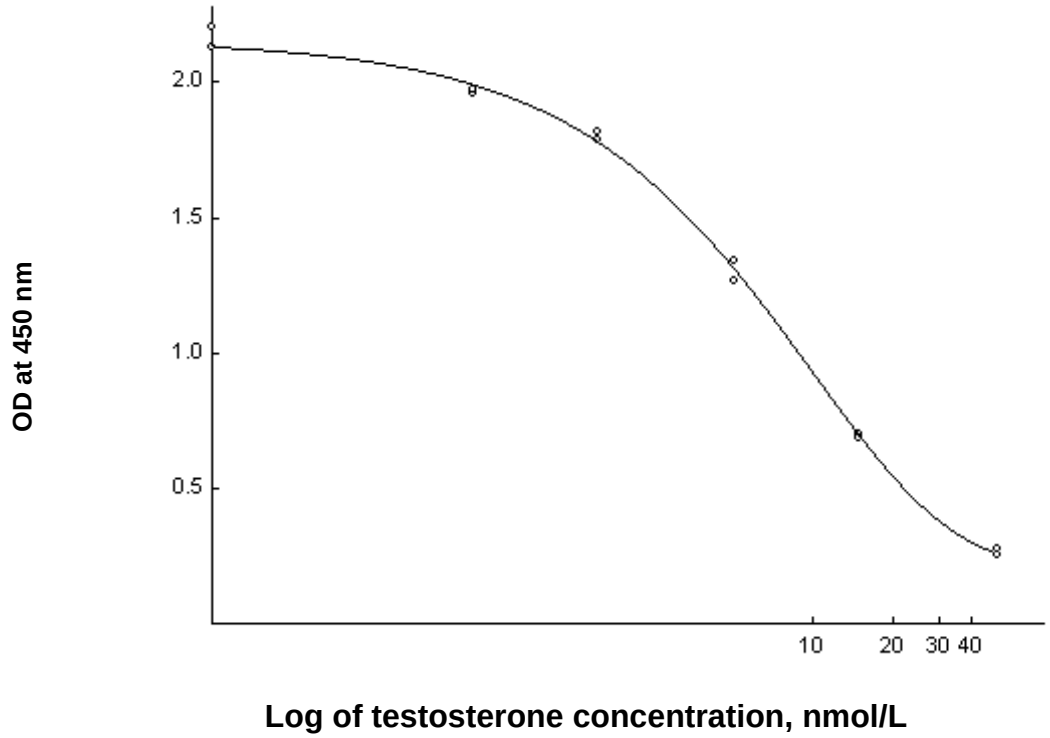


Fig. 2. Example of typical standard curve.

Do not use for evaluation of real assay data!

13.5. Assay scheme with shaking

Wells / Reagents	«Blank»	CAL / CONTROL	Samples
CAL / CONTROL	–	50 µL	–
Samples	–	–	50 µL
CONJ	–	150 µL	150 µL
Incubation No.1	90 min, +18…+25 °C, 500–800 rpm		
WASH P (diluted)	4 x 300 µL		
SUB	100 µL	100 µL	100 µL
Incubation No.2	15-30 min, +18…+25 °C, in the dark		
	10 min, +37 °C, 500–800 rpm		
STOP	100 µL	100 µL	100 µL
Stirring	1–2 min, +18…+25 °C		
OD measuring	450 nm		
Calculations	Corresponding software		

13.6. Assay scheme without shaking

Wells / Reagents	«Blank»	CAL / CONTROL	Samples
CAL / CONTROL	–	50 µL	–
Samples	–	–	50 µL
CONJ	–	150 µL	150 µL
Incubation No.1	90 min, +18…+25 °C (pre-shake for 1-2 minutes at room temperature)		
WASH P (diluted)	4 x 300 µL		
SUB	100 µL	100 µL	100 µL
Incubation No.2	15-30 min, +18…+25 °C, in the dark		
STOP	100 µL	100 µL	100 µL
Stirring	1–2 min, +18…+25 °C		
OD measuring	450 nm		
Calculations	Corresponding software		

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