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AccuBind
ELISA Microwells

TESTOSTERONE
Product Code: 3725-300

Intended Use: The Quantitative Determination of Total Testosterone Concentration in Human Serum or Plasma by a Microplate Enzyme Immunoassay

SUMMARY AND EXPLANATION OF THE TEST

Testosterone, (17β-Hydroxy-4-androstene-3-one), a C_{19} steroid, is the most potent naturally secreted androgen[1]. In normal post pubertal males, testosterone is secreted primarily by the testes with only a small amount derived from peripheral conversion of 4-Androstene-3, 17-dione (ASD)[2]. In adult women, it has been estimated that over 50% of serum testosterone is derived from peripheral conversion of ASD secreted by the adrenal and ovary, with the remainder from direct secretion of testosterone by these glands

In the male, testosterone is mainly synthesized in the interstitial Leydig cells and the testis, and is regulated by the interstitial cell stimulating hormone (ICSH), or luteinizing hormone (LH) of the anterior pituitary (the female equivalent of ICSH)[3]. Testosterone is responsible for the development of secondary sex characteristics, such as the accessory sex organs, the prostate, seminal vesicles and the growth of facial, pubic and auxiliary hair. Testosterone measurements have been very helpful in evaluating hypogonadal states. Increased testosterone levels in males can be found in complete androgen resistance (testicular feminization). Common causes of decreased testosterone levels in males include: hypogonadism, orchidectomy, estrogen therapy, Klinefelter's syndrome, hypopituitarism, and hepatic cirrhosis[2-4].

In the female, testosterone levels are normally found to be much lower than those encountered in the healthy male.
Testosterone in the female comes from three sources. It is secreted in small quantities by both the adrenal glands and the ovaries, and in healthy women 50–60% of the daily testosterone production arises from peripheral metabolism of prohormone, chiefly androstenedione. Common causes of increased serum testosterone levels in females include polycystic ovaries (Stein-Leventhal syndrome), ovarian tumors, adrenal tumors and adrenal hyperplasia. Virilization in women is associated with the administration of androgens and endogenous overproduction of testosterone. There appears to be a correlation between serum testosterone levels and the degree of virilization in women, although approximately 25% of women with varying degrees of virilism have serum testosterone levels that fall within the female reference range.

PRINCIPLE

Competitive Enzyme Immunoassay (TYPE 7):

The essential reagents required for a enzyme immunoassay include antibody, enzyme-antigen conjugate and native antigen. Upon mixing biotinylated antibody, enzyme-antigen conjugate and a serum containing the native antigen, a competition reaction results between the native antigen and the enzyme-antigen conjugate for a limited number of antibody binding sites. The interaction is illustrated by the followed equation:

$$^{Enz}Ag + Ag + Ab_{Btn} \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} AgAb_{Btn} + {}^{Enz}AgAb_{Btn}$$

$Ab_{C.W}$ = Monospecific Immobilized Antibody (Constant Quantity)

Ag = Native Antigen (Variable Quantity)

^{Enz}Ag = Enzyme-antigen Conjugate (Constant Quantity)

$AgAb_{Btn}$ = Antigen-Antibody Complex

$^{Enz}Ag\ Ab_{Btn}$ = Enzyme-antigen Conjugate -Antibody Complex

k_a = Rate Constant of Association

k_{-a} = Rate Constant of Disassociation

$K = k_a / k_{-a}$ = Equilibrium Constant

A simultaneous reaction between the biotin attached to the antibody and the streptavidin immobilized on the microwell occurs. This effects the separation of the antibody bound fraction after decantation or aspiration.

$AgAb_{Btn} + {}^{Enz}AgAb_{Btn} + \underline{Streptavidin_{CW}} \Rightarrow$ immobilized complex

$\underline{Streptavidin_{CW}}$ = Streptavidin immobilized on well

Immobilized complex = sandwich complex bound to the solid surface

The enzyme activity in the antibody bound fraction is inversely proportional to the native antigen concentration. By utilizing several different serum references of known antigen concentration, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.

REAGENTS MATERIALS PROVIDED:

A. Human Serum References -- 1ml/vial - Icons A-G
Seven (7) vials of serum reference for Testosterone at concentrations of 0 (**A**), 0.1 (**B**), 0.5 (**C**), 1.0 (**D**), 2.5 (**E**), 5.0 (**F**) and 12.0 (**G**) in ng/ml Store at 2-8°C. A preservative has been added. The calibrators can be expressed in molar concentrations (nM/L) by multiplying by 3.47.

For example: 1ng/ml x 3.47 = 3.47 nM/L

B. Testosterone Enzyme Reagent – 1.0 ml/vial Ⓔ
One (1) vial of Testosterone (Analog)-horseradish peroxides (HRP) conjugate in a protein stabilizing matrix with blue dye. Store at 2-8°C.

C. Testosterone Conjugate Buffer – 7.0 ml/vial - Icon Ⓑ
One (1) vial of reagent contains buffer, red dye, preservative, and binding protein inhibitors. Store at 2-8°C.

D. Testosterone Biotin Reagent—6.0 ml - Icon ∇
One (1) bottle of reagent contains anti-Testosterone biotinylated purified rabbit IgG conjugate in buffer, yellow dye and preservative. Store at 2-8°C.

⇓

E. Streptavidin Coated Plate -- 96 wells –Icon
One 96-well microplate coated with 1.0 µg/ml streptavidin and packaged in an aluminum bag with a drying agent. Store at 2-8°C.

F. Wash Solution -- 20ml - Icon
One (1) vial containing a surfactant in buffered saline. A preservative has been added. Store at 2-30°C.

G. Substrate A --7ml/vial - Icon S^A
One (1) vial contains tetramethylbenzidine (TMB) in buffer. Store at 2-8°C.

H. Substrate B -- 7ml/vial - Icon S^B
One (1) vial contains hydrogen peroxide (H_2O_2) in buffer. Store at 2-8°C.

I. Stop Solution -- 8ml/vial - Icon STOP
One (1) vial contains a strong acid (1N HCl). Store at 2-30°C.

J Product Instructions.

Note 1: Do not use reagents beyond the kit expiration date.
Note 2: Opened reagents are stable for sixty (60) days when stored at 2-8°C.
Note 3: Above reagents are for a single 96-well microplate.

Required But Not Provided:

1. Pipette capable of delivering 10µl, 50µl, and 100µl volumes with a precision of better than 1.5%.
2. Dispenser(s) for repetitive deliveries of 0.100ml and 0.300ml volumes with a precision of better than 1.5%.
3. Adjustable volume (200-1000µl) dispenser(s) for conjugate.
4. Microplate washer or a squeeze bottle (optional).
5. Microplate Reader with 450nm and 620nm wavelength absorbance capability.
6. Absorbent Paper for blotting the microplate wells.
7. Plastic wrap or microplate cover for incubation steps.
8. Vacuum aspirator (optional) for wash steps.
9. Timer.
10. Quality control materials.

PRECAUTIONS

For In Vitro Diagnostic Use
Not for Internal or External Use in Humans or Animals

All products that contain human serum have been found to be non-reactive for Hepatitis B Surface Antigen, HIV 1&2 and HCV Antibodies by FDA required tests. Since no known test can offer complete assurance that infectious agents are absent, all human serum products should be handled as potentially hazardous and capable of transmitting disease. Good laboratory procedures for handling blood products can be found in the Center for Disease Control / National Institute of Health, "Biosafety in Microbiological and Biomedical Laboratories," 2nd Edition, 1988, HHS Publication No. (CDC) 88-8395.

SPECIMEN COLLECTION AND PREPARATION

The specimens shall be blood; serum or plasma in type and the usual precautions in the collection of venipuncture samples should be observed. For accurate comparison to established normal values, a fasting morning serum sample should be obtained. The blood should be collected in a plain redtop venipuncture tube without additives or anti-coagulants (for serum) or evacuated tube(s) containing EDTA or heparin.. Allow the blood to clot for serum samples. Centrifuge the specimen to separate the serum or plasma from the cells.

Samples may be refrigerated at 2-8°C for a maximum period of five (5) days. If the specimen(s) cannot be assayed within this time, the sample(s) may be stored at temperatures of -20°C for up to 30 days. Avoid repetitive freezing and thawing. When assayed in duplicate, 0.020ml of the specimen is required.

REAGENT PREPARATION

1. **Working Enzyme Reagent -** Stable for 1 year.
   Measure 0.7 ml of 'Testosterone Enzyme Reagent' and add to the vial containing Testosterone Conjugate Buffer. Store at 2-8°C.

2. **Wash Buffer**
   Dilute contents of wash solution to 1000ml with distilled or deionized water in a suitable storage container. Diluted buffer can be stored at room temperature (20-27°C) for up to 60 days.

3. **Working Substrate Solution -** Stable for 1 year.
   Pour the contents of the amber vial labeled Solution 'A' into the clear vial labeled Solution 'B'. Place the yellow cap on the clear vial for easy identification. Mix and label accordingly. Store at 2 - 8°C.

Note: Do not use the working substrate if it looks blue.

TEST PROCEDURE

Before proceeding with the assay, bring all reagents, serum references and controls to room temperature (20 - 27°C).

1. Format the microplates' wells for each serum reference, control and patient specimen to be assayed in duplicate. **Replace any unused microwell strips back into the aluminum bag, seal and store at 2-8°C.**
2. Pipette 0.010 ml (10µL) of the appropriate serum reference, control or specimen into the assigned well.
3. Add 0.050 ml (50µl) of the working Testosterone Enzyme Reagent to all wells (see Reagent Preparation Section)..
4. Swirl the microplate gently for 20-30 seconds to mix.
5. Add 0.050 ml (50µl) of Testosterone Biotin Reagent to all wells.
6. Swirl the microplate gently for 20-30 seconds to mix.
7. Cover and incubate for 60 minutes at room temperature.
8. Discard the contents of the microplate by decantation or aspiration. If decanting, blot the plate dry with absorbent paper.
9. Add 300µl of wash buffer (see Reagent Preparation Section), decant (tap and blot) or aspirate. Repeat two (2) additional times for a total of three (3) washes. **An automatic or manual plate washer can be used. Follow the manufacturer's instruction for proper usage. If a squeeze bottle is employed, fill each well by depressing the container (avoiding air bubbles) to dispense the wash. Decant the wash and repeat two (2) additional times.**
10. Add 0.100 ml (100µl) of working substrate solution to all wells (see Reagent Preparation Section). **Always add reagents in the same order to minimize reaction time differences between wells.**
    DO NOT SHAKE THE PLATE AFTER SUBSTRATE ADDITION
11. Incubate at room temperature for fifteen (15) minutes.
12. Add 0.050ml (50µl) of stop solution to each well and gently mix for 15-20 seconds. **Always add reagents in the same order to minimize reaction time differences between wells.**
13. Read the absorbance in each well at 450nm (using a reference wavelength of 620-630nm to minimize well imperfections) in a microplate reader. **The results should be read within thirty (30) minutes of adding the stop solution.**

Note: Dilute the samples suspected of concentrations higher than 12 ng/ml 1:5 and 1:10 with Testosterone '0' ng/ml calibrator or female patient sera with a known low value for testosterone.

QUALITY CONTROL

Each laboratory should assay controls at levels in the low, normal and high range for monitoring assay performance. These controls should be treated as unknowns and values determined in every test procedure performed. Quality control charts should be maintained to follow the performance of the supplied reagents. Pertinent statistical methods should be employed to ascertain trends. The individual laboratory should set acceptable assay performance limits. In addition, maximum absorbance should be consistent with past experience. Significant deviation from established performance can indicate unnoticed change in experimental conditions or degradation of kit reagents. Fresh reagents should be used to determine the reason for the variations.

CALCULATION OF RESULTS

A dose response curve is used to ascertain the concentration of Testosterone in unknown specimens.

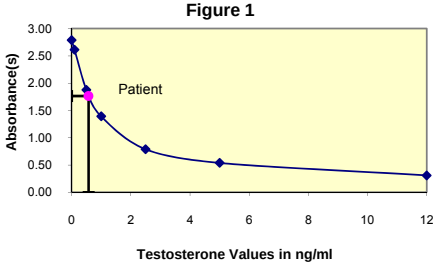
1. Record the absorbance obtained from the printout of the microplate reader as outlined in Example 1.
2. Plot the absorbance for each duplicate serum reference versus the corresponding Testosterone concentration in ng/ml on linear graph paper (do not average the duplicates of the serum references before plotting).
3. Connect the points with a best-fit curve.
4. To determine the concentration of Testosterone for an unknown, locate the average absorbance of the duplicates for each unknown on the vertical axis of the graph, find the intersecting point on the curve, and read the concentration (in ng/ml) from the horizontal axis of the graph (the duplicates of the unknown may be averaged as indicated). In the following example, the average absorbance (1. 764) intersects the dose response curve at (0.57ng/ml) Testosterone concentration (See Figure 1).

EXAMPLE 1

Sample I.D.	Well Number	Abs (A)	Mean Abs (B)	Value (ng/ml)
Cal A	A1	2.780	2.787	0
	B1	2.794		
Cal B	C1	2.576	2.611	0.1
	D1	2.646		
Cal C	E1	1.789	1.877	0.5
	F1	1.965		
Cal D	G1	1.391	1.392	1.0
	H1	1.393		
Cal E	A2	0.780	0.788	2.5
	B2	0.796		
Cal F	C2	0.530	0.538	5.0
	D2	0.547		
Cal G	E2	0.301	0.308	12.0
	F2	0.314		
Ctrl 1	G2	1.040	0.760	**1.61**
	H2	1.045		
Patient	A3	1.751	1.764	**0.57**
	B3	1.778		

*The data presented in Example 1 and Figure 1 is for illustration only and **should not** be used in lieu of a standard curve prepared with each assay.

Figure 1



Q.C. PARAMETERS

In order for the assay results to be considered valid the following criteria should be met:

1. The absorbance (OD) of calibrator 0 ng/ml should be ≥ 1.3.
2. Four out of six quality control pools should be within the established ranges.

LIMITATIONS OF PROCEDURE

A. Assay Performance

1. It is important that the time of reaction in each well is held constant for reproducible results.
2. Pipetting of samples should not extend beyond ten (10) minutes to avoid assay drift.
3. If more than one (1) plate is used, it is recommended to repeat the dose response curve.
4. Addition of the substrate solution initiates a kinetic reaction, which is terminated by the addition of the stop solution. Therefore, the addition of the substrate and the stopping solution should be added in the same sequence to eliminate any time-deviation during reaction.
5. Plate readers measure vertically. Do not touch the bottom of the wells.
6. Failure to remove adhering solution adequately in the aspiration or decantation wash step(s) may result in poor replication and spurious results.
7. Use components from the same lot. No intermixing of reagents from different batches.

B. Interpretation

1. If computer controlled data reduction is used to interpret the results of the test, it is imperative that the predicted values for the calibrators fall within 10% of the assigned concentrations.

EXPECTED RANGES OF VALUES

In agreement with established reference intervals[5] for a "normal" adult population, the expected ranges for the Testosterone AccuBind™ ELISA Test System are detailed in Table 1.

TABLE I
Expected Values for the Testosterone EIA Test System (ng/ml)

Male	**2.5 – 10.0**
Female	**0.2 – 0.95**

It is important to keep in mind that establishment of a range of values which can be expected to be found by a given method for a population of "normal" persons is dependent upon a multiplicity of factors: the specificity of the method, the population tested and the precision of the method in the hands of the analyst. For these reasons each laboratory should depend upon the range of expected values established by the Manufacturer only until an in-house range can be determined by the analysts using the method with a population indigenous to the area in which the laboratory is located.

PERFORMANCE CHARACTERISTICS

A. Precision

The within and between assay precision of the Testosterone AccuBind™ Microplate EIA Test System were determined by analyses on three different levels of pool control sera. The number, mean values, standard deviation and coefficient of variation for each of these control sera are presented in Table 2 and Table 3.

TABLE 2
Within Assay Precision (Values in ng/ml)

Sample	N	X	σ	C.V.
Low	22	1.61	0.07	4.5%
Normal	22	4.86	0.25	5.2%
High	22	8.19	0.59	7.3%

TABLE 3
Between Assay Precision (Values in ng/ml)

Sample	N	X	σ	C.V.
Low	10	1.47	0.09	6.3%
Normal	10	4.90	0.29	5.9%
High	10	8.99	0.54	6.0%

*As measured in ten experiments in duplicate over a ten day period.

B. Accuracy

The Testosterone AccuBind™ Microplate EIA Test System was compared with a chemiluminescence immunoassay method. Biological specimens from low, normal and high Testosterone level populations were used (The values ranged from 0.29 ng/ml – 21.9ng/ml). The total number of such specimens was 58. The least square regression equation and the correlation coefficient were computed for this Testosterone EIA in comparison with the reference method. The data obtained is displayed in Table 4.

TABLE 4

Method	Mean (x)	Least Square Regression Analysis	Correlation Coefficient
This Method (y)	3.12	Y= -0.265+0.944(X)	0.985
Reference (X)	3.02		

Only slight amounts of bias between this method and the reference method are indicated by the closeness of the mean values. The least square regression equation and correlation coefficient indicates excellent method agreement.

C. Sensitivity

The Testosterone AccuBind™ Microplate EIA Test System has a sensitivity of 0.38pg. This is equivalent to a sample containing a concentration of 0.038 ng/ml. The sensitivity was ascertained by determining the variability of the 0 ng/ml serum calibrator and using the 2σ (95% certainty) statistic to calculate the minimum dose.

D. Specificity

The % cross reactivity of the testosterone antibody to selected substances was evaluated by adding the interfering substance to a serum matrix at various concentrations. The cross-reactivity was calculated by deriving a ratio between dose of interfering substance to dose of Testosterone needed to displace the same amount of labeled analog.

Substance	Cross Reactivity
Testosterone	1.0000
Androstenedione	0.0009
Dihydotestosterone	0.0178
Cortisone	<0.0001
Corticosterone	<0.0001
Cortisol	<0.0001
Spirolactone	<0.0001
Progesterone	<0.0001
17α-OH Progesterone	<0.0001
DHEA sulfate	<0.0001
Estradiol	<0.0001
Estrone	<0.0001
Estriol	<0.0001

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Size		96(A)	192(B)
Reagent (fill)	A)	1ml set	1ml set
	B)	1 (1ml)	2 (1ml)
	C)	1 (7ml)	2 (7ml)
	D)	1 (7ml)	2 (7ml)
	E)	1 plate	2 plates
	F)	1 (20ml)	1 (20ml)
	G)	1 (7ml)	2 (7ml)
	H)	1 (7ml)	2 (7ml)
	I)	1 (8ml)	2 (8ml)

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