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ELISA PRODUCT INFORMATION & MANUAL

Testosterone ELISA Kit (Colorimetric)

NBP2-42044

Enzyme-linked Immunosorbent Assay for quantitative detection of Testosterone. For research use only. Not for diagnostic or therapeutic procedures.

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DESCRIPTION

The Testosterone ELISA kit is a competitive immunoassay for the quantitative determination of Testosterone in biological fluids. Please read the complete kit insert before performing this assay. This kit uses a monoclonal antibody to Testosterone to bind, in a competitive manner, Testosterone in the standard or sample or an alkaline phosphatase molecule which has Testosterone covalently attached to it. After simultaneous incubations at room temperature the excess reagents are washed away and substrate is added. After a short incubation time the enzyme reaction is stopped and the yellow color generated is read on a microplate reader at 405nm. The intensity of the bound yellow color is inversely proportional to the concentration of Testosterone in either standards or samples. The measured optical density is used to calculate the concentration of Testosterone. For further explanation of the principles and practice of immunoassays please see the excellent books by Chard¹ or Tijssen².

INTRODUCTION

Testosterone is one of the most important androgens secreted into the bloodstream and is synthesized from pregnenolone which is itself formed from cholesterol³⁻⁶. In adult humans approximately 5 mg of testosterone are synthesized per day and circulate in plasma predominately bound to proteins, including specific sex hormone binding globulin (SHBG) and nonspecific proteins such as albumin. It is believed that the bioavailable testosterone includes the free steroid and the albumin bound steroid and these equal about 35% of the total testosterone⁵. Both testosterone and SHBG exhibit rhythmic variations. In serum, testosterone concentration peaks between 4am and 8am, while SHBG concentration is effected by such factors as posture^{7,8}. Testosterone is the main androgen secreted by the Leydig cells of the testes and effects both primary and secondary sexual development such as muscle mass and sex drive^{9,10} OH

Testosterone







SAFETY WARNINGS & PRECAUTIONS

DIAGNOSTIC PROCEDURES.

FOR RESEARCH USE ONLY. NOT FOR USE IN

- 2. Stop Solution is a solution of trisodium phosphate. This solution is caustic; care should be taken in use.
- The activity of the alkaline phosphatase conjugate is dependent on the presence of Mg²⁺ and Zn²⁺ ions. The activity of the conjugate is affected by concentrations of chelators (>10 mM) such as EDTA and EGTA.
- 4. We test this kit's performance with a variety of samples, however it is possible that high levels of interfering substances may cause variation in assay results.
- The Testosterone Standard provided, is supplied in ethanolic buffer at a pH optimized to maintain Testosterone integrity. Care should be taken handling this material because of the known and unknown effects of steroids.

Avoid freeze /

thaw cycles

MATERIALS SUPPLIED

1. Goat anti-Mouse IgG Microtiter Plate, One Plate of 96 Wells

A plate using break-apart strips coated with goat antibody specific to mouse IgG.

- Testosterone ELISA Conjugate, 5 mL
 A blue solution of alkaline phosphatase conjugated with Testosterone.
- Testosterone ELISA Antibody, 5 mL A yellow solution of a mouse monoclonal antibody to Testosterone.

4. Assay Buffer 3, 27 mL

Tris buffered saline containing proteins and detergents and sodium azide as a preservative.

5. Wash Buffer Concentrate, 27 mL

6. Tris buffered saline containing detergents.

7. Testosterone Standard, 0.5 mL

A solution of 50,000 pg/mL Testosterone.

 Steroid Displacement Reagent, 1 mL A specially formulated displacer to inhibit steroid binding to proteins.

9. pNpp Substrate, 20 mL

A solution of p-nitrophenyl phosphate in buffer. Ready to use.

10. Stop Solution, 5 mL

A solution of trisodium phosphate in water. Keep tightly capped. Caution: Caustic.

- 11. Testosterone Assay Layout Sheet, 1 each
- 12. Plate Sealer, 1 each

STORAGE

All components of this kit are stable at 4°C until the kit's expiration date.

OTHER MATERIALS NEEDED

- 1. Deionized or distilled water.
- 2. Precision pipets for volumes between 5 μ L and 1,000 μ L.
- 3. Repeater pipets for dispensing 50 μ L and 200 μ L.
- 4. Disposable beaker for diluting buffer concentrates.
- 5. Graduated cylinders.
- 6. A microplate shaker.
- 7. A 37°C Incubator.
- 8. Adsorbent paper for blotting.
- 9. Microplate reader capable of reading at 405 nm, preferably with correction between 570 and 590 nm.

SAMPLE HANDLING

The Testosterone ELISA kit is compatible with Testosterone samples in a wide range of matrices. Samples diluted sufficiently into Assay Buffer 3 can be read directly from the standard curve. Please refer to the Sample Recovery recommendations on page 15 for details of suggested dilutions. However, the end user must verify that the recommended dilutions are appropriate for their samples. Included with the kit is the Steroid Displacement Reagent which should be added to serum, plasma and other samples containing steroid binding proteins. Samples should be diluted with 1 part of the Reagent for every 99 parts of sample. Samples containing mouse IgG may interfere with the assay.

Human Serum and Plasma samples are not suitable for this kit.

Samples in the majority of Tissue Culture Media can also be read in the assay provided the standards have been diluted into the Tissue Culture Media instead of Assay Buffer 3. There will be a small change in the binding associated with running the standards and samples in media. Users should only use standard curves generated in media or buffer to calculate concentrations of Testosterone in the appropriate matrix.

Some samples may have very low levels of Testosterone present and extraction may be necessary for accurate measurement. A suitable extraction procedure is outlined below:

Materials Needed

- 1. Testosterone Standard to allow extraction efficiency to be accurately determined.
- 2. ACS Grade Diethyl Ether.

Procedure

- 1. Add sufficient Testosterone to a typical sample for determination of extraction efficiency.
- 2. In a fume hood add 1 mL of Diethyl Ether for every mL of sample. Stopper and shake sample.
- 3. Allow layers to separate. Carefully pipet off the top ether layer and place in a clean test tube.
- 4. Repeat steps 1 and 2 twice more, combining the ether layers.

- 5. Evaporate the ether to dryness under nitrogen.
- Dissolve the extracted Testosterone with at least 250 µL of Assay Buffer 3 by vortexing well then allow to sit for five minutes at room temperature. Repeat twice more.
- 7. Run the reconstituted samples in the assay immediately or keep dried samples frozen below -20°C in desiccation.

PROCEDURAL NOTES

- 1. Do not mix components from different kit lots or use reagents beyond the kit expiration date.
- Allow all reagents to warm to room temperature for at least 30 minutes before opening.
- 3. Standards can be made up in either glass or plastic tubes.
- 4. Pre-rinse the pipet tip with reagent, use fresh pipet tips for each sample, standard and reagent.
- 5. Pipet standards and samples to the bottom of the wells.
- 6. Add the reagents to the side of the well to avoid contamination.
- 7. This kit uses break-apart microtiter strips, which allow the user to measure as many samples as desired. Unused wells must be kept desiccated at 4°C in the sealed bag provided. The wells should be used in the frame provided.
- 8. Care must be taken to minimize contamination by endogenous alkaline phophatase. Contaminating alkaline phosphatase activity, especially in the substrate solution, may lead to high blanks. Care should be taken not to touch pipet tips and other items that are used in the assay with bare hands.
- 9. Prior to addition of substrate, ensure that there is no residual wash buffer in the wells. Any remaining wash buffer may cause variation in assay results.

REAGENT PREPARATION

1. Testosterone Standard

Allow the 50,000 pg/mL Testosterone standard solution to warm to room temperature. Label five 12 x 75 mm glass tubes #1 through #5. Pipet 1 mL of standard diluent (Assay Buffer 3 or Tissue Culture Media) into tube #1. Pipet 750 μ L of standard diluent into tubes #2 through #5. Remove 40 μ L of diluent from tube #1. Add 40 μ L of the 50,000 pg/mL standard to tube #1. Vortex thoroughly. Add 250 μ L of tube #1 to tube #2 and vortex thoroughly. Add 250 μ L of tube #2 to tube #3 and vortex. Continue this for tubes #4 and #5.

The concentration of Testosterone in tubes #1 through #5 will be 2,000, 500, 125, 31.25 and 7.81 pg/mL respectively. See the Testosterone Assay Layout Sheet for dilution details.

Diluted standards should be used within 60 minutes of preparation.

2. Wash Buffer

Prepare the Wash Buffer by diluting 5 mL of the supplied concentrate with 95 mL of deionized water. This can be stored at room temperature until the kit expiration date, or for 3 months, whichever is earlier.

ASSAY PROCEDURE

Bring all reagents to room temperature for at least 30 minutes prior to opening.

All standards and samples should be run in duplicate.

- Refer to the Assay Layout Sheet to determine the number of wells to be used and put any remaining wells with the desiccant back into the pouch and seal the ziploc. Store unused wells at 4°C.
- Pipet 100 µL of standard diluent (Assay Buffer 3 or Tissue Culture Media) into the NSB and the Bo (0 pg/mL Standard) wells.
- 3. Pipet 100 µL of Standards #1 through #5 into the appropriate wells.
- 4. Pipet 100 μ L of the Samples into the appropriate wells.
- 5. Pipet 50 µL of Assay Buffer 3 into the NSB wells.

- Pipet 50 μL of yellow Antibody into each well, except the Blank, TA and NSB wells.
- Incubate the plate at room temperature on a plate shaker for 1 hour at ~500 rpm.
- Pipet 50 μL of blue Conjugate into each well, except the Total Activity (TA) and Blank wells.

NOTE: Every well used should be Green in color except the NSB wells which should be Blue. The Blank and TA wells are empty at this point and have no color.

- Incubate the plate at room temperature on a plate shaker for 1 hour at ~500 rpm
- Empty the contents of the wells and wash by adding 400 μL of wash solution to every well. Repeat the wash 2 more times for a total of 3 washes.
- 11. After the final wash, empty or aspirate the wells, and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
- 12. Add 5 μ L of the blue Conjugate to the TA wells.
- Add 200 µL of the pNpp Substrate solution to every well. Incubate at 37°C for 1 hour without shaking. The plate must be covered with the plate sealer provided.
- 14. Add 50 μL of Stop Solution to every well. This stops the reaction and the plate should be read immediately.
- 15. Blank the plate reader against the Blank wells, read the optical density at 405 nm, preferably with correction between 570 and 590 nm. If the plate reader is not able to be blanked against the Blank wells, manually subtract the mean optical density of the Blank wells from all readings.

CALCULATION OF RESULTS

Several options are available for the calculation of the concentration of Testosterone in the samples. We recommend that the data be handled by an immunoassay software package utilizing a 4 parameter logistic curve fitting program. If data reduction software is not readily available, the concentration of Testosterone can be calculated as follows:

 Calculate the average net Optical Density (OD) bound for each standard and sample by subtracting the average NSB OD from the average OD bound:

Average Net OD = Average OD - Average NSB OD

 Calculate the binding of each pair of standard wells as a percentage of the maximum binding wells (Bo), using the following formula:

Percent Bound = <u>Net OD</u> x 100 Net Bo OD

TYPICAL RESULTS

The results shown below are for illustration only and **should not** be used to calculate results from another assay.

Sample	Mean OD (-Blank)	Average Net OD	Percent Bound	Testosterone (pg/mL)
Blank OD	(0.177)			
ТА	0.173			
NSB	0.005	0.000	0%	
Во	0.447	0.442	100%	0
S1	0.043	0.038	8.6%	2,000
S2	0.115	0.110	24.9%	500
S3	0.257	0.252	57.0%	125
S4	0.387	0.382	86.4%	31.25
S 5	0.428	0.423	95.7%	7.81
Unknown 1	0.069	0.064	14.5%	994
Unknown 2	0.384	0.379	85.7%	30.9

TYPICAL STANDARD CURVES

Typical standard curves are shown below. These curves must not be used to calculate **Testosterone** concentrations; each user must run a standard curve for each assay.



TYPICAL QUALITY CONTROL PARAMETERS

Total Activity Added	=	0.173 x 10 = 1.73
%NSB	=	0.289%
%Bo/TA	=	25.5%
Quality of Fit parameter logistic curv	= /e fit)	0.9999 (calculated from a 4
20% Intercent	_	646 ng/ml

=	040 pg/mL
=	166 pg/mL
=	46 pg/mL
	= =

PERFORMANCE CHARACTERISTICS

The following parameters for this kit were determined using the guidelines listed in the National Committee for Clinical Laboratory Standards (NCCLS) Evaluation Protocols12.

Sensitivity

Sensitivity was calculated in Assay Buffer 3, using the two hour primary incubation, by determining the average optical density bound for twenty (20) wells run as Bo, and comparing to the average optical density for twenty (20) wells run with Standard #5. The detection limit was determined as the concentration of Testosterone measured at two (2) standard deviations from the zero along the standard curve.

Average Optical I	Density for	the Bo =	0.431 ± (0.008 (1.9%)		
Average Optical Density for Standard #5 = 0.409 ± 0.009 (2.20						
Delta Optical Der	nsity (0-7.8	pg/mL) =		0.022		
2 SD's of the Zero Standard = $2 \times 0.008 = 0.016$						
Sensitivity =	<u>0.016</u> x 0.022	7.8 pg/mL	=	5.67 pg/mL		

Linearity

A sample containing 236.1 pg/mL Testosterone was diluted 4 times 1:2 in the kit Assay Buffer 3 and measured in the assay. The data was plotted graphically as actual Testosterone concentration versus measured Testosterone concentration.

The line obtained had a slope of 0.975 and a correlation coefficient of 0.999.

Precision

Intra-assay precision was determined by taking samples containing low, medium and high concentrations of Testosterone and running these samples multiple times (n=8) in the same assay. Inter-assay precision was determined by measuring three samples with low, medium and high concentrations of Testosterone in multiple assays (n=8).

The precision numbers listed below represent the percent coefficient of variation for the concentrations of Testosterone determined in these assays as calculated by a 4 parameter logistic curve fitting program.

	Testosterone (pg/mL)	Intra-assay %CV	Inter-assay %CV
Low	38.3	10.8	
Medium	91.0	10.0	
High	269.9	7.8	
Low	32.0		14.6
Medium	81.2		11.3
High	259.1		9.3

Cross Reactivities

The cross reactivities for a number of related steroid compounds was determined by dissolving the cross reactant (purity checked by N.M.R. and other analytical methods) in Assay Buffer 3 at concentrations from 50,000 to 10 pg/mL. These samples were then measured in the Testosterone assay, and the measured Testosterone concentration at 50% B/Bo was calculated. The % cross reactivity was calculated by comparison with the actual concentration of cross reactant in the sample and expressed as a percentage.

Chemical Name	Common Name	Cross- reactivity
4-androsten-17β-ol-3- one	Testosterone	100%
4-androsten-17β, 19- diol-3-one	19-hydroxytestosterone	14.64%
4-androsten-3,17-dione	Androstendione	7.20%
5-androsten-3β-ol-17- one	Dehydroepiandrosteron e	0.72%
1,3,5(10)-estratrien- 3,17β-diol	Estradiol	0.40%
5α-androsten-17β-ol-3- one	Dihydrotestosterone	<0.001%
1,3,5(10)-estratrien- 3,16α,17β-triol	Estriol	<0.001%
4-pregen-11β,21-diol- 3,18,20-trione	Aldosterone	<0.001%
4-pregen-11β,21-diol- 3,20-dione	Corticosterone	<0.001%
4-pregen-11β,17α,21- triol-3, 20-dione	Cortisol	<0.001%
4-pregan-17α,21-diol- 3,11,20-trione	Cortisone	<0.001%
1,3,5(10)-estratrien-3-ol- 17-one	Estrone	<0.001%
4-pregen-3,20-dione	Progesterone	<0.001%
5-pregen-3β-ol-20-one	Pregenolone	<0.001

SAMPLE RECOVERIES

Please refer to pages 6 and 7 for Sample Handling recommendations and Standard preparation.

Testosterone concentrations were measured in a variety of different samples including tissue culture media, human saliva and porcine serum and plasma. Testosterone was spiked into the undiluted samples of these media which were then diluted with the appropriate diluent and assayed in the kit. The following results were obtained::

Sample	% Recovery*	Recommended Dilution*
Tissue Culture Media	104.1	1:2
Human Saliva	100.9	1:20
Porcine Serum	102.7	1:20
Porcine EDTA	100.9	1:20
Plasma		

* See Sample Handling instructions on page 6 for details.

REFERENCES

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NOTES

TESTOSTERONE ASSAY LAYOUT SHEET

Std.	Diluent Vol. (µL)	Vol. Added (µL)	Testosterone Conc. (pg/mL)
1	960	40, Stock	2,000
2	750	250, Std. 1	500
3	750	250, Std. 2	125
4	750	250, Std. 3	31.25
5	750	250, Std. 4	7.8

• DILUTION TABLE FOR MAKING STANDARDS 1-5:

• ASSAY PROTOCOL FLOW CHART:

Well I.D.:	Blank A1, B1	TA C1, D1	NSB E1, F1	Bo G1, H1	Stds. A2 - B3	Samples C3 - H12
Standard Diluent			100 µL	100 µL		
Assay Buffer 3			50 µL			
Std. and/or Sample					100 µL	100 µL
Antibody				50 µL	50 µL	50 µL
Incub. 1 hour @ RT, shaking	$\Rightarrow\Rightarrow\Rightarrow\Rightarrow\Rightarrow$	$\Rightarrow\Rightarrow\Rightarrow\Rightarrow\Rightarrow$	$\Rightarrow \Rightarrow \Rightarrow \Rightarrow \Rightarrow$	$\Rightarrow\Rightarrow\Rightarrow\Rightarrow\Rightarrow$	$\Rightarrow\Rightarrow\Rightarrow\Rightarrow\Rightarrow$	$\Rightarrow\Rightarrow\Rightarrow\Rightarrow\Rightarrow$
Conjugate			50 µL	50 µL	50 µL	50 µL
Incub. 1 hour @ RT, shaking	$\Rightarrow\Rightarrow\Rightarrow\Rightarrow$	$\Rightarrow\Rightarrow\Rightarrow\Rightarrow\Rightarrow$	$\Rightarrow \Rightarrow \Rightarrow \Rightarrow$	$\Rightarrow\Rightarrow\Rightarrow\Rightarrow\Rightarrow$	$\Rightarrow\Rightarrow\Rightarrow\Rightarrow$	$\Rightarrow\Rightarrow\Rightarrow\Rightarrow\Rightarrow$
Asp. & Wash 3 x 400 µL	$\Rightarrow\Rightarrow\Rightarrow\Rightarrow\Rightarrow$	$\Rightarrow \Rightarrow \Rightarrow \Rightarrow$	$\Rightarrow \Rightarrow \Rightarrow \Rightarrow \Rightarrow$	$\Rightarrow\Rightarrow\Rightarrow\Rightarrow\Rightarrow$	$\Rightarrow \Rightarrow \Rightarrow \Rightarrow \Rightarrow$	$\Rightarrow\Rightarrow\Rightarrow\Rightarrow\Rightarrow$
Conjugate		5 µL				
Substrate	200 µL	200 µL	200 µL	200 µL	200 µL	200 µL
Incub. 1 hour @ 37°C, sealed	$\Rightarrow\Rightarrow\Rightarrow\Rightarrow\Rightarrow$	$\Rightarrow\Rightarrow\Rightarrow\Rightarrow\Rightarrow$	$\Rightarrow \Rightarrow \Rightarrow \Rightarrow$	$\Rightarrow \Rightarrow \Rightarrow \Rightarrow$	$\Rightarrow \Rightarrow \Rightarrow \Rightarrow \Rightarrow$	$\Rightarrow\Rightarrow\Rightarrow\Rightarrow\Rightarrow$
Stop Solution	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL

• TESTOSTERONE PLATE LAYOUT:

A1 Blank	A2 Std 1	A3 Std 5	A4	A5	A6	A7	A8	A9	A10	A11	A12
ві Blank	в2 Std 1	вз Std 5	В4	B5	B6	B7	B8	B9	B10	B11	B12
cı TA	c2 Std 2	С3	C4	C5	C6	C7	C8	С9	C10	C11	C12
di TA	D2 Std 2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12
e1 NSB	E2 Std 3	E3	E4	E5	E6	E7	E8	Е9	E10	E11	E12
F1 NSB	F2 Std 3	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
gi Bo	G2 Std 4	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
ні Во	н2 Std 4	НЗ	H4	Н5	Н6	Н7	H8	Н9	H10	H11	H12

	Exp. Date		Date	Tech
Start Time	Temp 1	Notes:		
End Time	Temp			
Start Time	Temp			
End Time	Temp			
	Start Time End Time Start Time End Time	Exp. Date Start Time Temp. End Time Temp. Start Time Temp. End Time Temp.	Exp. DateStart TimeTemp.End TimeTemp.Start TimeTemp.End TimeTemp.	Exp. Date Date Start Time Temp. Notes: End Time Temp.