Enzo

Product Manual

Testosterone ELISA kit

Catalog #: ADI-900-065

96 Well Kit

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Product Manual

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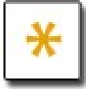

Please read entire booklet before proceeding with the assay.

Please contact Enzo Life Sciences Technical Support if necessary.

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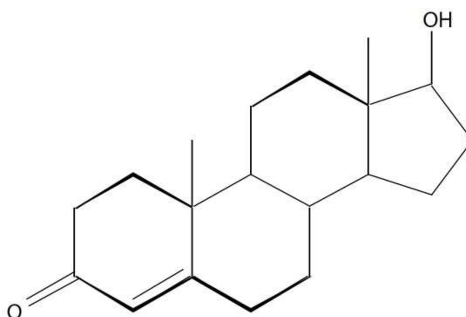
DESCRIPTION

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 Chard[1] or Tijssen[2].

INTRODUCTION

Testosterone is one of the most important androgens secreted into the bloodstream and is synthesized from pregnenolone which is itself formed from cholesterol[3-6]. 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 testosterone[5]. Both testosterone and SHBG exhibit rhythmic variations. In serum, testosterone concentration peaks between 4am and 8am, while SHBG concentration is affected 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].

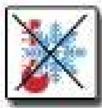
Testosterone



SAFETY WARNINGS & PRECAUTIONS

FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.

Handle with care


Avoid freeze / thaw cycles

1. Some kit components contain azide, which may react with lead or copper plumbing. When disposing of reagents always flush with large volumes of water to prevent azide build-up.
2. Stop Solution is a solution of trisodium phosphate. This solution is caustic; care should be taken in use.
3. The activity of the alkaline phosphatase conjugate is dependent on the presence of Mg^{2+} and Zn^{2+} 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.
5. The Testosterone Standard provided, Catalog No. 80-0430, 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.

MATERIALS SUPPLIED

1. **Goat anti-Mouse IgG Microtiter Plate, One Plate of 96 Wells, Component number 80-0050**

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

2. **Testosterone ELISA Conjugate, 5 mL, Component number 80-0431**

   A blue solution of alkaline phosphatase conjugated with Testosterone.

3. **Testosterone ELISA Antibody, 5 mL, Component number 80-0429**

   A yellow solution of a mouse monoclonal antibody to Testosterone.

4. **Assay Buffer 3, 27 mL, Component number 80-0145**

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

5. **Wash Buffer Concentrate, 27 mL, Component number 80-1286**

   Tris buffered saline containing detergents.

6. **Testosterone Standard, 0.5 mL, Component number 80-0430**

   A solution of 50,000 pg/mL Testosterone.

7. **Steroid Displacement Reagent, 1 mL, Component number 80-0120**

   A specially formulated displacer to inhibit steroid binding to proteins.

8. **pNpp Substrate, 20 mL, Component number 80-0075**

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

9. **Stop Solution, 5 mL, Component number 80-0247**

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

10. **Testosterone Assay Layout Sheet, 1 each, Component number 30-0092**

11. **Plate Sealer, 1 each, Component number 30-0012**

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 µL and 1,000 µL.
3. Repeater pipets for dispensing 50 µL and 200 µL.
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.

6. 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.
2. 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 960 µL of standard diluent (Assay Buffer 3 or Tissue Culture Media) into tube #1.

   Pipet 750 µL of standard diluent into tubes #2 through #5.

   Add 40 µL of the 50,000 pg/mL standard to tube #1. Vortex thoroughly.

   Add 250 µL of tube #1 to tube #2 and vortex thoroughly. Add 250 µL 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.

1. 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.
2. 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 µL of the Samples into the appropriate wells.

5. Pipet 50 µL of Assay Buffer 3 into the NSB wells.
6. Pipet 50 µL of yellow Antibody into each well, except the Blank, TA and NSB wells.
7. Incubate the plate at room temperature on a plate shaker for 1 hour at ~500 rpm.
8. 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.
9. Incubate the plate at room temperature on a plate shaker for 1 hour at ~500 rpm
10. 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 µL of the blue Conjugate to the TA wells.
13. 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:

1. 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

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

   $$\text{Percent Bound} = \frac{\text{Net OD}}{\text{Net Bo OD}} \times 100$$

3. Plot the average Net OD versus concentration of Testosterone for the standards in order to generate a standard curve. Fit a line through the data points (4PL curve fit is suggested). The concentration of Testosterone in the samples can be determined by interpolation off of the standard curve.

Samples that read outside of the standard curve range will need to be re-analyzed using a different dilution, or will require further concentrating of the sample during the extraction protocol.

Be sure to multiple sample concentrations by the dilution factor used during sample preparation. If samples were extracted, the amount they were concentrated during extraction must also be taken into account.

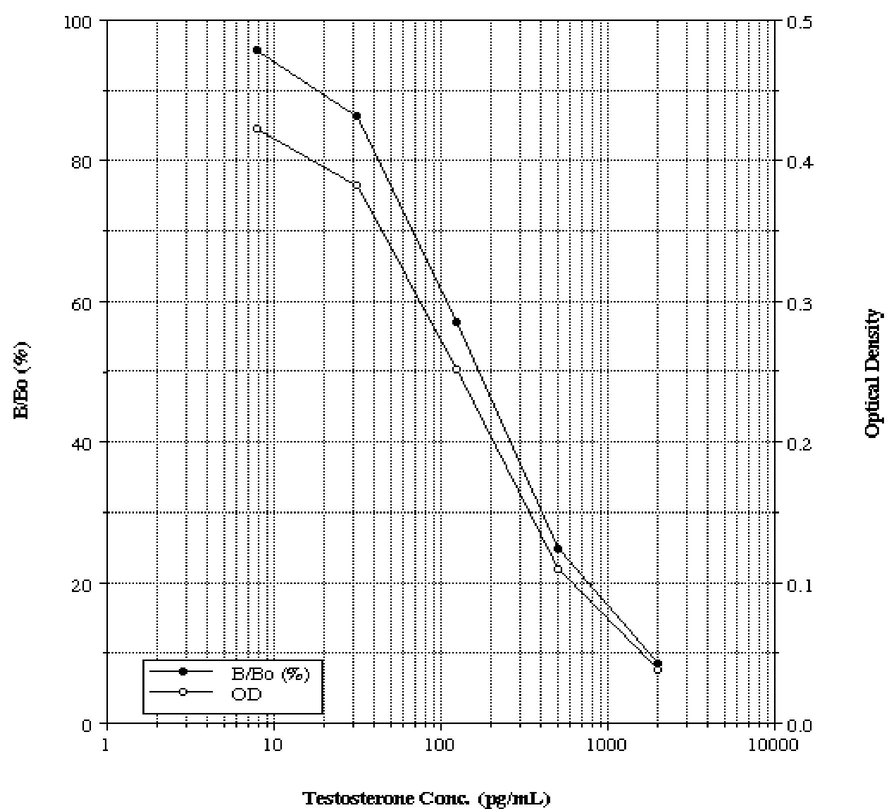
TYPICAL RESULTS

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)			
TA	0.173			
NSB	0.005	0.000	0%	
Bo	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
S5	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

Quality of Fit = 0.9999 (calculated from a 4 parameter logistic curve fit)

20% Intercept = 646 pg/mL

50% Intercept = 166 pg/mL

80% Intercept = 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 Density for the Bo = 0.431 ± 0.008 (1.9%)

Average Optical Density for Standard #5 = 0.409 ± 0.009 (2.2%)

Delta Optical Density (0-7.8 pg/mL) = 0.022

2 SD's of the Zero Standard = 2 x 0.008 = 0.016

$$\text{Sensitivity} = \frac{0.016}{0.022} \times 7.8 \text{ pg/mL} = 5.67 \text{ pg/mL}$$

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	Dehydroepiandrosterone	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 Plasma	100.9	1:20

* See Sample Handling instructions on page 6 for details.

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NOTES

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