

Testosterone high sensitivity ELISA Kit

Catalog #: ADI-900-176

96 well Enzyme-linked Immunosorbent Assay Kit

For use with serum, plasma and urine

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Technical Support (US): 800-942-0430
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Product Manual

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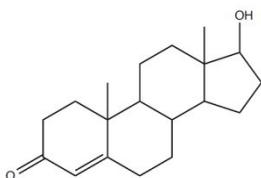


Carefully note the handling and storage conditions of each kit component.

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INTRODUCTION

Testosterone is one of the most important androgens secreted into the bloodstream and is synthesized from pregnenolone which is itself formed from cholesterol¹⁻⁴. 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 affected by such factors as posture^{5,6}. 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^{7,8}.



PRINCIPLE OF THE ASSAY

1. Standards and samples are added to wells coated with a GxM IgG antibody. A blue solution of Testosterone conjugated to alkaline phosphatase is then added, followed by a yellow solution of mouse monoclonal antibody to testosterone.
2. During a simultaneous incubation at room temperature the antibody binds in a competitive manner, the testosterone in the sample or conjugate. The plate is washed, leaving only bound testosterone.
3. pNpp substrate solution is added. The substrate generates a yellow color when catalyzed by the alkaline phosphatase on the testosterone conjugate.
4. Stop solution is added. The yellow color is read at 405 nm. The amount of signal is indirectly proportional to the amount of testosterone in the sample.

MATERIALS SUPPLIED

- 1. Assay Buffer Low BSA**
27 mL, Component number 80-2079
Tris buffer containing proteins and sodium azide
- 2. Testosterone Standard**
0.5 mL, Component number 80-0430
A solution of 50,000 pg/mL Testosterone
- 3. Goat anti-Mouse IgG Microtiter Plate**
One plate of 96 wells, Component number 80-0050
A clear plate of break-apart strips coated with a goat anti-mouse polyclonal antibody
- 4. Testosterone Antibody**
5 mL, Component number 80-2241
A yellow solution of a monoclonal antibody to Testosterone
- 5. Testosterone Conjugate**
5 mL, Component number 80-2242
A blue solution of Testosterone conjugated to alkaline phosphatase
- 6. Wash Buffer Concentrate**
27 mL, Component number 80-1286
Tris buffered saline containing detergents
- 7. pNpp Substrate**
20 mL, Component number 80-0075
A solution of p-nitrophenyl phosphate
- 8. Stop Solution**
5 mL, Component number 80-0247
A solution of trisodium phosphate in water
- 9. Testosterone Assay Layout Sheet**
1 each, Component number 30-0288
- 10. Plate Sealer**
1 each, Component number 30-0012



Do not mix components from different kit lots or use reagents beyond the expiration date of the kit.



The standard should be handled with care due to the known and unknown effects of the antigen.



Avoid contamination by endogenous alkaline phosphatase. Do not expose reagents or supplies to bare skin.



Activity of conjugate is affected by concentrations of chelators > 10mM (such as EDTA and EGTA).



Stop solution is caustic. Keep tightly capped.



Storage temp

STORAGE

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

ADDITIONAL MATERIALS NEEDED BUT NOT SUPPLIED

1. Deionized or distilled water
2. Precision pipets for volumes between 5 μ L and 1,000 μ L
3. Repeater pipet for dispensing 50 μ L and 200 μ L
4. Disposable beakers for diluting buffer concentrates
5. Graduated cylinders
6. Microplate shaker
7. Lint-free paper toweling for blotting
8. Microplate reader capable of reading at 405 nm
9. 200 mg C₁₈ solid phase system columns (Burdick & Jackson recommended)
10. Vacuum manifold
11. Speedvac
12. 100% Methanol
13. Diethyl Ether
14. Data reduction software capable of analyzing data, preferably with a 4 parameter logistic curve fit

SAMPLE HANDLING



If buffers other than those provided are used in the assay, the end-user must determine the appropriate dilution and assay validation.

For an accurate determination of testosterone concentration in serum, plasma and urine samples, extract samples prior to assaying. A minimum 1:4 dilution is required for all samples after extraction. These are the minimum dilutions required to remove matrix interference in the assay.

Samples with low levels of testosterone may be concentrated during the extraction procedure. For example, extracting 1 mL of sample and reconstituting with 0.25 mL of the assay buffer would concentrate the sample 4 times.

Either liquid-liquid extraction or solid phase extraction methods may be used. Solid phase extraction allows for higher throughput sample processing with the use of a vacuum manifold and speedvac.

Solid Phase Extraction

Materials Needed:

1. Testosterone standard to allow extraction efficiency to be accurately determined. An extraction efficiency protocol is available on our website.
2. 200 mg C₁₈ solid phase system columns (Burdick & Jackson recommended)
3. Vacuum manifold
4. Speedvac
5. 100% Methanol
6. Diethyl Ether
7. dH₂O



Samples must be stored at or below -20°C to avoid loss of bioactive analyte. Repeated freeze/thaw cycles should be avoided.

Procedure

1. Condition 200 mg C₁₈ solid phase system columns on a vacuum manifold by passing 5-10 mL of 100% methanol through the columns, followed by 5-10 mL of dH₂O.
2. Apply serum and plasma samples.
3. Wash columns with 5-10 mL dH₂O. Allow water to drain completely from columns until dry.
4. Elute samples with 2 mL of diethyl ether.
5. Dry samples down in a speedvac for 2-3 hrs.
6. Rehydrate samples at room temperature in the assay buffer. A minimum of 250 µL of the assay buffer is recommended for reconstitution to allow for duplicate sample measurement.

Liquid-Liquid Extraction

Materials Needed:

1. Testosterone standard to allow extraction efficiency to be accurately determined. An extraction efficiency protocol is available on our website.
2. Diethyl Ether
3. Speedvac
4. dH₂O

Procedure

1. Add diethyl ether to serum or plasma samples at a 5:1 (v/v) ether:sample ratio.
2. Mix solutions by vortexing for 2 minutes.
3. Allow phases to separate for 2 minutes.
4. Transfer organic phase to a glass test tube containing 1 mL dH₂O.
5. Vortex the mixture for 2 minutes, and allow phases to separate for 2 minutes.
6. Transfer organic phase to a clean glass test tube, and dry samples down using a speed vac for 2-3 hours.
7. Rehydrate samples at room temperature in the assay buffer. A minimum of 250 µL of the assay buffer is recommended for reconstitution to allow for duplicate sample measurement



Tip: Place samples in the freezer for a few minutes to help the layers separate.

SAMPLE RECOVERIES

Testosterone was spiked into samples at high, medium and low concentrations, prior to liquid-liquid extractions. Endogenous testosterone was subtracted from the spiked values and the average recovery in each of the spiked matrices was compared to the recovery of identical spikes in extracted assay buffer. The mean and the range percent recovery at the three concentrations are indicated below for each matrix.

Sample Matrix	Dilution	Spike Concentration	Recovery of Spike (Range)
Human Plasma (n=5)	1:16	1000 pg/mL	84% (78-96%)
		200 pg/mL	86% (55-117%)
		50 pg/mL	118% (62-141%)
Human Serum (n=5)	1:16	1000 pg/mL	84% (70-93%)
		200 pg/mL	97% (66-145%)
		50 pg/mL	111% (63-115%)
Human Urine (n=1)	1:16	1000 pg/mL	111%
		200 pg/mL	82%
		50 pg/mL	117%

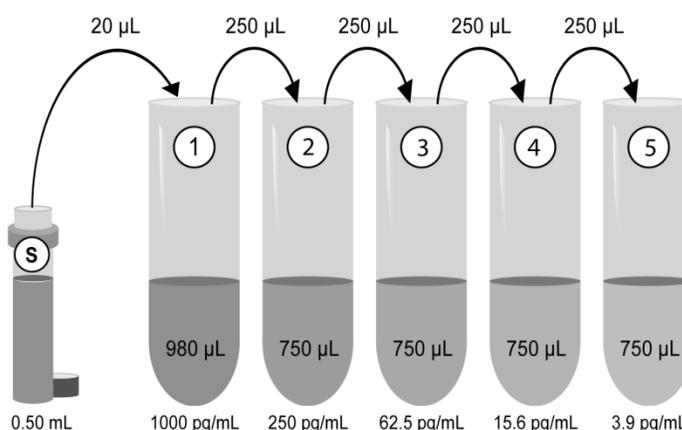
REAGENT PREPARATION

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

 Glass or polypropylene tubes may be used for standard preparation. Avoid polystyrene.

- 1. Wash Buffer**
 Prepare the wash buffer by diluting 5 mL of the supplied Wash Buffer Concentrate with 95 mL of deionized water. This can be stored at room temperature until the kit expiration, or for 3 months, whichever is earlier.
- 2. Testosterone Standard**
 Allow the 50,000 pg/mL Testosterone standard to come to room temperature and vortex prior to use. Label five 12 x 75mm tubes #1 through #5. Pipet 1mL assay buffer into tube #1. Pipet 750 µL assay buffer into tubes #2 through #5. Remove 20 µL of assay buffer from tube #1. Add 20 µL of the 50,000 pg/mL standard stock to tube #1 and vortex thoroughly. Add 250 µL from tube #1 and add to tube #2. Vortex thoroughly. Continue this for tubes #3 through #5.

 Prior to the addition of substrate, ensure there is no residual wash buffer in the wells. Remaining wash buffer may cause variation in assay results.



Diluted standards should be used within 60 minutes of preparation. The concentrations of Testosterone in the tubes are labeled above.

ASSAY PROCEDURE

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

 Pre-rinse each pipet tip with reagent. Use fresh pipet tips for each sample, standard, and reagent.

 All standards and samples should be run in duplicate.

 Pipet the reagents to the sides of the wells to avoid possible contamination.

Refer to the Assay Layout Sheet to determine the number of wells to be used. Remove the wells not needed for the assay and return them, with the desiccant, to the mylar bag and seal. Store unused wells at 4°C.

1. Pipet 150 µL of the assay buffer into the NSB (non-specific binding) wells.
2. Pipet 100 µL of the assay buffer into the Bo (0 pg/mL standard) wells.
3. Pipet 100 µL of Standards #1 through #5 to the bottom of the appropriate wells.
4. Pipet 100 µL of the samples to the bottom of the appropriate wells.
5. Pipet 50 µL of the blue conjugate into each well except the TA and Blank wells.
6. Pipet 50 µL of the yellow antibody into each well except the Blank, TA, and NSB 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.

7. Seal the plate. Incubate at room temperature with shaking (~500 rpm*) for two hours.
8. Empty the contents of the wells and wash by adding 400 µL of wash buffer to every well. Repeat 2 more times for a total of **3 washes**. 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.
9. Pipet 5 µL of the blue conjugate to the TA wells.
10. Add 200 µL of the substrate solution into each well.

11. Incubate for 1 hour at room temperature with shaking.
12. Pipet 50 μ L stop solution into each well.
13. After blanking the plate reader against the substrate blank, read optical density at 405 nm. If plate reader is not capable of adjusting for the blank, manually subtract the mean OD of the substrate blank from all readings.

* The optimal speed for each shaker will vary and may range from 120-700 rpm. The speed must be set to ensure adequate mixing of the wells, but not so vigorously that the contents of the wells splash out and contaminate other wells.

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 OD for each standard and sample by subtracting the average NSB OD from the average OD for each standard and sample.

$$\text{Average Net OD} = \text{Average OD} - \text{Average NSB OD}$$

2. Calculate the binding of each pair of standard wells as a percentage of the maximum binding wells (B_0), using the following formula:

$$\text{Percent Bound} = \frac{\text{Net OD}}{\text{Net } B_0 \text{ OD}} \times 100$$

3. Plot the Percent Bound (B/B_0) versus concentration of Testosterone for the standards. Approximate a straight line through the points. The concentration of Testosterone of the unknowns can be determined by interpolation.

Samples with concentrations outside of the standard curve range will need to be re-analyzed using a different dilution.

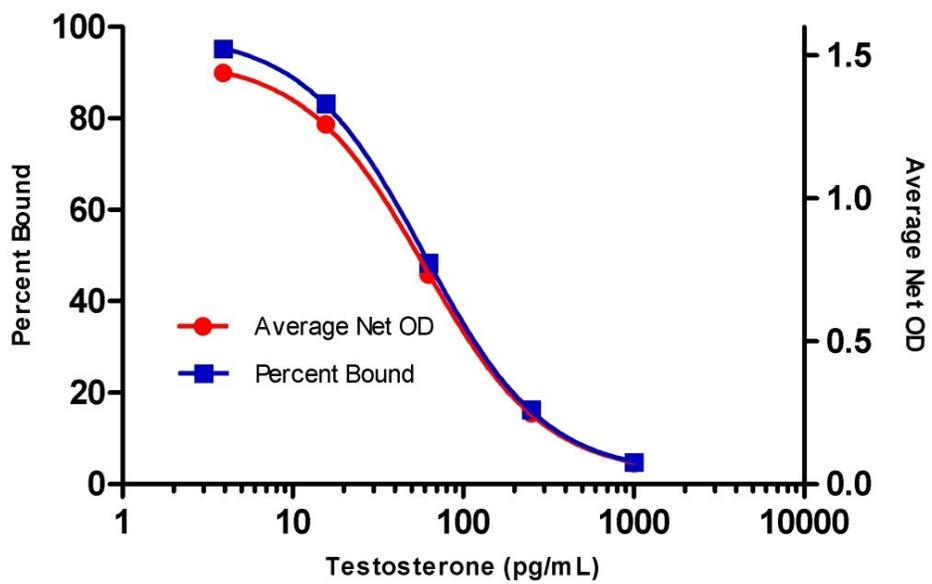


Make sure to adjust sample concentrations by the dilution or concentration factor used during sample preparation.

TYPICAL RESULTS

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

Sample	Average Net OD	Percent Bound	Testosterone
TA	0.436	---	---
NSB	0.095	0%	---
Bo	1.511	100%	0
S1	0.074	4.8%	1000
S2	0.249	16.3%	250
S3	0.734	48.4%	62.5
S4	1.259	83.2%	15.6
S5	1.439	95.2%	3.9



PERFORMANCE CHARACTERISTICS

Specificity

The cross reactivities for a number of related compounds were determined by diluting the cross reactants in the kit assay buffer at a concentration of one hundred times the high standard. These samples were then measured in the assay.

Compound	Cross Reactivity
Androstenedione	16.4%
19-Hydroxytestosterone	7.6%
Dihydrotestosterone	2.7%
Progesterone	0.3%
Androsterone	0.2%
DHEA	0.22%
Estrone	0.09%
Estradiol	<0.004%
Estriol	<0.004%
Epitestosterone	<0.004%
Hydrocortisone-21 hemi-succinate	<0.004%

Sensitivity

The sensitivity or limit of detection of the assay is 2.6 pg/mL. The sensitivity was determined by interpolation at 2 standard deviations below the mean signal at maximal binding (0 pg/mL) using data from 7 standard curves.

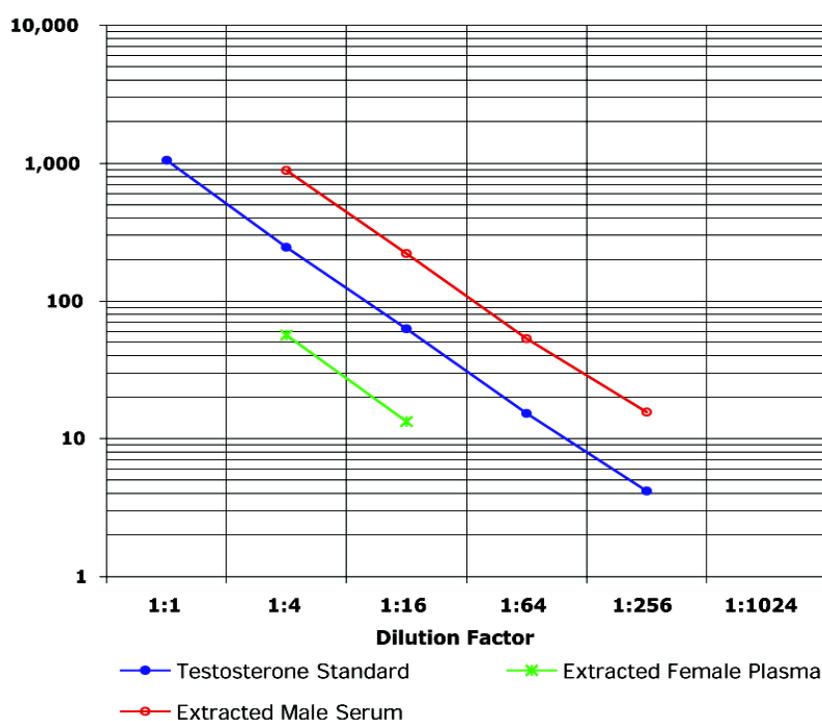
Linearity

Human serum, plasma and urine samples were serially diluted 1:4 in the kit assay buffer and measured in the assay. The results are shown in the table below.

Dilution	Average % of Expected		
	Serum	Plasma	Urine
Neat	---	---	---
1:4	104	103	108
1:16	104	94	100
1:64	100	106	99
1:256	116	100	87

Parallelism

Dose-response curves from extracted male serum and extracted female plasma diluted into assay buffer were compared to the Testosterone standard curve. The parallel response indicates the standard effectively mimics the native protein.



Precision

Intra-assay precision was determined by assaying 20 replicates of three buffer controls containing Testosterone in a single assay.

pg/mL	% CV
374.9	4.1
79.70	2.3
43.05	3.6

Inter-assay precision was determined by measuring buffer controls (n=13) of varying Testosterone concentrations in multiple assays over several days.

pg/mL	% CV
388.8	9.9
86.8	10.2
43.0	7.4

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Product Manual

GLOBAL HEADQUARTERS

Enzo Life Sciences Inc.
10 Executive Boulevard
Farmingdale, NY 11735
Toll-Free: 1.800.942.0430
Phone: 631.694.7070
Fax: 631.694.7501
info-usa@enzolifesciences.com

EUROPE

Enzo Life Sciences (ELS) AG
Industriestrasse 17
CH-4415 Lausen
Switzerland
Phone: +41/0 61 926 89 89
Fax: +41/0 61 926 89 79
info-ch@enzolifesciences.com

For local distributors and detailed product information visit us online:
www.enzolifesciences.com