Testosterone ELISA Kit Instructions

Please read all instructions carefully before beginning this assay

PRODUCT #402510

For research use only.

Storage Conditions:
Do not freeze kit components
All other kit components: 2-8°C

DESCRIPTION

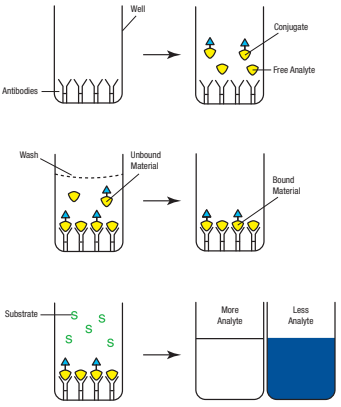
Testosterone is the principle androgen. It is synthesized in the testis, the ovary and the adrenal cortex. It is responsible for the development and maintenance of the male secondary sex characteristics. It also exerts important protein anabolic and growth-promoting effects. The plasma testosterone levels are useful in investigating hypogonadism and hormone replacement therapy in men. It is also useful as a marker in hyper-androgenism in women.

PRINCIPLE OF ASSAY

This is an ELISA (Enzyme-Linked ImmunoSorbent Assay) for the quantitative analysis of Testosterone levels in biological fluid. This test kit operates on the basis of competition between the enzyme conjugate and the Testosterone in the sample for a limited number of binding sites on the antibody coated plate.

The sample or standard solution is first added to the microplate. Next, the diluted enzyme conjugate is added and the mixture is shaken and incubated at room temperature for one hour. During the incubation, competition for binding sites is taking place. The plate is then washed removing all the unbound material. The bound enzyme conjugate is detected by the addition of substrate which generates an optimal color after 30 minutes. Quantitative test results may be obtained by measuring and comparing the absorbance reading of the wells of the samples against the standards with a microplate reader at 650 nm. The extent of color development is inversely proportional to the amount of Testosterone in the sample or standard. For example, the absence of Testosterone in the sample will result in a bright blue color, whereas the presence of Testosterone will result in decreased or no color development.

PRINCIPLE OF ASSAY (continued)



MATERIALS PROVIDED

1. EIA BUFFER: 30 mL. Provided to dilute enzyme conjugate and Testosterone standards.
2. WASH BUFFER (10X): 20 mL. Dilute 10-fold with deionized water. Diluted wash buffer is used to wash all unbound enzyme conjugate, samples and standards from the plate after the one hour incubation.
3. K-BLUE SUBSTRATE: 20 mL. Stabilized 3,3', 5,5' Tetramethylbenzidine (TMB) plus Hydrogen Peroxide (H_2O_2) in a single bottle. It is used to develop the color in the wells after they have been washed. Keep substrate refrigerated. LIGHT SENSITIVE.
4. EXTRACTION BUFFER (5X): 30 mL. Dilute 5-fold with deionized water. This buffer is used for diluting extracted and non-extracted samples.
5. TESTOSTERONE ENZYME CONJUGATE: 150 µL. Testosterone horseradish peroxidase concentrate. Blue capped vial.
6. TESTOSTERONE STANDARD: 100 µL. Testosterone standard provided at the concentration of 1 µg/mL. Green capped vial.
7. TESTOSTERONE ANTIBODY-COATED MICROPLATE: A 96 well MaxiSorp™ Nunc microplate with anti-Testosterone rabbit antibody precoated on each well. The plate is ready for use as is. DO NOT WASH!

MATERIALS NEEDED BUT NOT PROVIDED

1. 300 mL deionized water to dilute wash buffer and extraction buffer.
2. Precision pipettes that range from 10 µL-1000 µL and disposable tips.

NOTE: If all or several strips are to be used at one time, it is suggested that a multichannel pipette be used.

3. Clean test tubes used to dilute the standards and conjugate.
4. Graduated cylinders to dilute and mix wash buffer and extraction buffer.
5. Microplate reader with 650 nm filter.
6. Plate cover or plastic film to cover plate during incubation.

OPTIONAL MATERIALS:

7. 1 N HCl or Neogen's Red Stop Solution.
8. Microplate shaker.

If performing an extraction on samples, the following will be required:

9. Ethyl ether or ethyl acetate
10. Nitrogen gas
11. Vortex

WARNINGS AND PRECAUTIONS

1. DO NOT use components beyond expiration date.
2. DO NOT mix any reagents or components of this kit with any reagents or components of any other kit. This kit is designed to work properly as provided.
3. DO NOT pipette reagents by mouth.
4. Always pour substrate out of the bottle into a clean test tube - DO NOT pipette out of the bottle. If the pipette tip is unclean this could result in contamination of the substrate.
5. All specimens should be considered potentially infectious. Exercise proper handling precautions.
6. DO NOT smoke, eat or drink in areas where specimens or reagents are being handled.
7. Use aseptic technique when opening and removing reagents from vials and bottles.
8. Keep plate covered except when adding reagents, washing or reading.
9. Kit components should be refrigerated at all times when not in use.

PROCEDURAL NOTES

1. It is not necessary to allow reagents to warm to room temperature before use.
2. Desiccant bag must remain in foil pouch with unused strips. Keep zip-lock pouch sealed when not in use to maintain a dry environment.
3. Always use new pipette tips to pipette buffer, enzyme conjugate, standards and samples.
4. Before pipetting a reagent, rinse the pipette tip three times with that reagent (i.e. fill the tip with the desired amount of reagent and dispense back into the same vial - repeat 2 times). Now the tip is properly rinsed and ready to dispense the reagent into your well or test tube.
5. When pipetting into the wells, DO NOT allow the pipette tip to touch the inside of the well, or any of the reagents already in the well. This can result in cross contamination.
6. Standards and samples should be assayed in duplicate.
7. To quantitate, always run samples alongside a standard curve. If testing a sample that is not extracted, standards should be diluted in the same type of medium being tested. This medium should be known to be negative.
8. Gently mix specimens and reagents before use. Avoid vigorous agitation.
9. When using only partial amounts of a kit, it is recommended to transfer the appropriate volume of each reagent to a clean vessel for repeated dispensing. This will reduce reagent contamination caused by repeated sampling from the original container.
10. The enzyme conjugate is most stable in its concentrated form. Dilute only the volume necessary for the amount of strips currently being used.
11. Before taking an absorbance reading wipe the outside bottom of the wells with a lint-free wiper to remove dust and fingerprints.
12. Before opening the enzyme conjugate and standard vial, tap vial in an upright position to remove any liquid in the cap.

SAMPLE PREPARATION

This assay is non-species specific. Usually, urine and tissue culture supernatant can be assayed directly by diluting them with the diluted extraction buffer. Plasma and most other mediums will need to be extracted.

EXTRACTION OF TESTOSTERONE

1. Pipette 100 µL of plasma into a glass tube (10x75 mm) and add 1 mL of ethyl ether.
2. Vortex the tube for 30 seconds and then allow the phases to separate.
3. Transfer the organic phase into a clean glass tube and evaporate the solvent with a stream of N_2.
4. Dissolve the residue in 100 µL of diluted extraction buffer.
5. Dilute the extract 100 fold by adding 10 µL of the above extract into 990 µL of diluted extraction buffer.
6. Vortex and assay 50 µL in duplicates.
7. The values obtained are multiplied by 100 to give final ng/mL concentrations.
8. If the concentration is higher than the high range of the standard curve, the samples in #6 need to be further diluted and reassayed.

NOTE: Extraction buffer must be diluted 5 fold with deionized water before use. Any precipitant present must be brought into solution before dilution.

TEST PROCEDURES

1. Prepare standards as follows:

Standard	Preparation
A	stock solution 1 µg/mL (Provided in green capped vial)
B	take 20 µL of A, add to 980 µL of EIA buffer and mix=20 ng/mL
C	take 200 µL of B, add to 1.8 mL of EIA buffer and mix=2 ng/mL
D	take 200 µL of C, add to 1.8 mL of EIA buffer and mix=0.2 ng/mL

Continue standard preparation following Scheme I.

SCHEME I

Standards	ng/mL	EIA buffer (µL added)	C standard µL	D standard µL
S_0	0	as is	-	-
S_1	0.002	990	-	10
S_2	0.004	980	-	20
S_3	0.008	960	-	40
S_4	0.02	900	-	100
S_5	0.04	800	-	200
S_6	0.08	600	-	400
S_7	0.2	-	-	as is

2. Determine the number of wells to be used.
3. Dilute the Testosterone enzyme conjugate. Add 1 µL of enzyme conjugate into 50 µL total volume of EIA buffer for each well assayed. For the whole plate, add 110 µL of the enzyme conjugate into 5.5 mL total volume of EIA buffer. Mix the solution thoroughly by inversion only.
4. Add 50 µL of standards (S) or unknown (U) (some samples may require diluting) to the appropriate wells in duplicate. See Scheme II for suggested template design.
5. Add 50 µL of the diluted enzyme conjugate to each well. Use 8-channel pipette or 12-channel pipette for rapid addition.
6. Mix by shaking plate gently. A microplate shaker may be used.
7. Cover plate with plastic film or plate cover and incubate at room temperature for one hour. NOTE: Keep plate away from drafts and temperature fluctuations.
8. Dilute concentrated wash buffer with deionized water (i.e. 20 mL of wash buffer plus 180 mL of deionized water). Mix thoroughly.
9. After incubation, dump out the contents of the plate. Tap out contents thoroughly on a clean lint-free towel.
10. Wash each well with 300 µL of the diluted wash buffer. Repeat for a total of three washings. An automated plate washer can be used, however, increase number of wash cycles from three to five with an automated plate washer.
11. Add 150 µL of substrate to each well. Use multichannel pipette for best results. Mix by shaking plate gently.
12. Incubate at room temperature for 30 minutes.
13. Gently shake plate before taking a reading to ensure uniform color throughout each well.
14. Plate is read in a microplate reader at 650 nm. If a dual wavelength is used, set W_1 at 650 nm and W_2 at 490 nm.
15. If accounting for substrate background, use 2 to 8 wells as blanks with only substrate in the wells (150 µL/well). Subtract the average of these absorbance values from the absorbance values of the wells being assayed.

NOTE: Some microplate readers can be programmed to do these subtractions automatically when reading the plate. Consult your instrument manual.

OPTIONAL TEST PROCEDURES

16. Add 50-100 µL of 1 N HCl or Neogen's Red Stop Solution to each well to stop enzyme reaction.
17. Read plate at 450 nm, if 1 N HCl solution was used. Read plate at 650 nm, if Neogen's Red Stop Solution was used.
18. Plot the standard curve and estimate the concentrations of the samples from the curve. See "CALCULATIONS."

NOTE: Absorbance readings will approximately double when stopped with acid. If absorbance readings are too high for measuring with your microplate reader, decrease the substrate incubation approximately 10 minutes but no more than 15 minutes.

SCHEME II

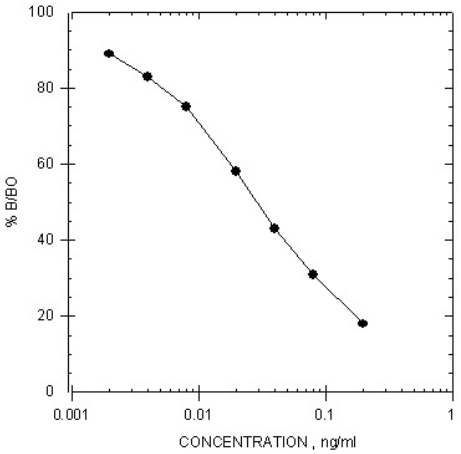
	1	2	3	4	5	6	7	8	9	10	11	12
A	$^{S}0$	$^{S}0$	$^{U}1$	$^{U}1$	$^{U}9$	$^{U}9$	$^{U}17$	$^{U}17$	$^{U}25$	$^{U}25$	$^{U}33$	$^{U}33$
B	$^{S}1$	$^{S}1$	$^{U}2$	$^{U}2$	$^{U}10$	$^{U}10$	$^{U}18$	$^{U}18$	$^{U}26$	$^{U}26$	$^{U}34$	$^{U}34$
C	$^{S}2$	$^{S}2$	$^{U}3$	$^{U}3$	$^{U}11$	$^{U}11$	$^{U}19$	$^{U}19$	$^{U}27$	$^{U}27$	$^{U}35$	$^{U}35$
D	$^{S}3$	$^{S}3$	$^{U}4$	$^{U}4$	$^{U}12$	$^{U}12$	$^{U}20$	$^{U}20$	$^{U}28$	$^{U}28$	$^{U}36$	$^{U}36$
E	$^{S}4$	$^{S}4$	$^{U}5$	$^{U}5$	$^{U}13$	$^{U}13$	$^{U}21$	$^{U}21$	$^{U}29$	$^{U}29$	$^{U}37$	$^{U}37$
F	$^{S}5$	$^{S}5$	$^{U}6$	$^{U}6$	$^{U}14$	$^{U}14$	$^{U}22$	$^{U}22$	$^{U}30$	$^{U}30$	$^{U}38$	$^{U}38$
G	$^{S}6$	$^{S}6$	$^{U}7$	$^{U}7$	$^{U}15$	$^{U}15$	$^{U}23$	$^{U}23$	$^{U}31$	$^{U}31$	$^{U}39$	$^{U}39$
H	$^{S}7$	$^{S}7$	$^{U}8$	$^{U}8$	$^{U}16$	$^{U}16$	$^{U}24$	$^{U}24$	$^{U}32$	$^{U}32$	$^{U}40$	$^{U}40$

CALCULATIONS

1. After the substrate background has been subtracted from all absorbance values, average all of your duplicate well absorbance values.
2. The average of your two S_0 values is now your B_0 value. (S_1 now becomes B_1, etc.)
3. Next, find the percent of maximal binding (%B/B_0 value). To do this, divide the averages of each standard absorbance value (now known as B_1 through B_7) by the B_0 absorbance value and multiply by 100 to achieve percentages.
4. Graph your standard curve by plotting the %B/B_0 for each standard concentration on the ordinate (y) axis against concentration on the abscissa (x) axis. Draw a curve by using a curve-fitting routine (i.e. 4-parameter or linear regression).
5. Divide the averages of each sample absorbance value by the B_0 value and multiply by 100 to achieve percentages.
6. Using the standard curve, the concentration of each sample can be determined by comparing the %B/B_0 of each sample to the corresponding concentration of Testosterone standard.
7. If the samples were diluted, the concentration determined from the standard curve must be multiplied by the dilution factor.

TYPICAL STANDARD CURVE

Testosterone in EIA Buffer



TYPICAL DATA

NOTE: "Typical data" is a representation. Variances in data will occur. Optical density readings may fluctuate during the shelf-life of the kit, but the $\%B/B_0$ should remain comparable. Measuring wavelength: 650 nm

Standard	Standard Concentration (ng/mL)	Optical Density (Absorbance Value)	$\%B/B_0$
S_0 (B_0)	0	1.152	100
S_1 (B_1)	0.002	1.023	89
S_2 (B_2)	0.004	0.959	83
S_3 (B_3)	0.008	0.861	75
S_4 (B_4)	0.02	0.672	58
S_5 (B_5)	0.04	0.497	43
S_6 (B_6)	0.08	0.352	31
S_7 (B_7)	0.2	0.202	18

CROSS REACTIVITY

TESTOSTERONE	100.0%
DIHYDROTESTOSTERONE	100.0%
TESTOSTERONE GLUCURONIDE	16.12%
ANDROSTENEDIONE	0.86%
BOLANDIOL	0.86%
TESTOSTERONE ENANTHATE	0.13%
ESTRIOL	0.10%
TESTOSTERONE BENZOATE	0.10%
ESTRADIOL	0.05%
DEHYDROEPIANDROSTERONE	0.04%
TESTOSTERONE PROPIONATE	0.04%
DEOXYCORTICOSTERONE	0.03%
TESTOSTERONE 17ß-CYPIONATE	0.02%
ALDOSTERONE	<0.01%
CORTICOSTERONE	<0.01%
CORTISOL	<0.01%
CORTISONE	<0.01%
ESTRONE	<0.01%
17-HYDROXYPROGESTERONE	<0.01%
PREGNENOLONE	<0.01%
PROGESTERONE	<0.01%

REFERENCES

1. Rehman, J., Christ, G., Allyskewycz, M., Kerr, E., and Melman, A. Experimental hyperprolactinemia in a rat model: alteration in centrally mediated neuroerectile mechanisms. International Journal of Impotence Research. 12, 23-32(2000).

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TECHNICAL ASSISTANCE

Technical assistance is available Monday-Friday, between 8:00 a.m. and 6:00 p.m. EST.

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