

Enzyme Immunoassay for Testosterone

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INTRODUCTION

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.

PRINCIPLES OF PROCEDURE

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 the substrate that generates an optimal color after 30 minutes. Quantitative test results may be obtained by measuring and comparing the absorbance reading of the sample wells against the wells of the standard curve 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.

MATERIALS PROVIDED

Component	Description	Volume	Storage
EIA Buffer	Buffer used to dilute the Conjugate and Testosterone Standards.	30 mL	4°C
10x Wash Buffer	Buffer used to wash the plate prior to color development.	20 mL	4°C
TMB Substrate	TMB substrate used for color development.	20 mL	4°C
5x Extraction Buffer	Buffer used to dilute extracted and non-extracted samples.	30 mL	4°C
Testosterone-HRP Conjugate	Testosterone horseradish peroxidase concentrated conjugate.	150 µL	4°C
Testosterone Standard	1 µg/mL Testosterone standard solution.	100 µL	4°C
Coated Plate	96-well microplate coated with rabbit anti-Testosterone antibody.	1 plate	4°C

MATERIALS NEEDED BUT NOT PROVIDED

1. Microplate reader with a 450 nm or 650 nm filter
2. Adjustable micropipettes (10 – 1000 µL) and tips
3. Deionized water
4. Plate cover or plastic film
5. Test tubes
6. 1 N HCl (optional)

EXTRACTION MATERIALS

1. Ethyl Ether
2. Nitrogen Gas
3. Vortex

STORAGE

1. Store the components of this kit at the temperatures specified on the labels.
2. Unopened reagents are stable until the indicated kit expiration date.
3. Desiccant bag must remain in foil pouch with unused strips. Keep pouch sealed when not in use to maintain a dry environment. Remove excess air before sealing.

WARNINGS AND PRECAUTIONS

1. Use aseptic technique when opening and dispensing reagents.
2. This kit is designed to work properly as provided and instructed. Additions, deletions or substitutions to the procedure or reagents are not recommended, as they may be detrimental to the assay.

PROCEDURAL NOTES

1. The enzyme conjugate is most stable in its concentrated form. Dilute only the volume necessary for the amount of strips currently being used.
2. To minimize errors in absorbance measurements due to handling, wipe the exterior bottom of the microplate wells with a lint-free paper towel prior to inserting into the plate reader.

SAMPLE PREPARATION

1. Urine and tissue culture supernatant can be assayed after diluting them with diluted Extraction Buffer.
2. Plasma and most other mediums will need to be extracted.

EXTRACTION PROTOCOL

1. Pipet 100 μ L of plasma into a glass test tube (10x75 mm) and add 1 mL of Ethyl Ether.
2. Vortex the tube for 30 seconds and allow the phases to separate.
3. Transfer the organic phase into a clean glass tube and evaporate the solvent with a Nitrogen stream.
4. Dissolve the residue in 100 μ L of diluted Extraction Buffer.
5. Dilute the extract 100 fold by adding 10 μ L of the above extract into 990 μ L of diluted Extraction Buffer.
6. If the concentration is higher than the high range of the standard curve, the samples will need to be further diluted and re-assayed.

REAGENT PREPARATION

1. **5x Extraction Buffer:** Dilute the appropriate amount to 1x with deionized water prior to use.
2. **10x Wash Buffer:** Add 20 mL of 10x Wash Buffer to 180 mL of deionized water prior to use.
3. **Testosterone-HRP Conjugate:** Dilute 110 μ L of Conjugate into 5.5 mL total volume of EIA Buffer.

STANDARD CURVE PREPARATION

The Testosterone Standard is provided as a 1 $\mu\text{g}/\text{mL}$ stock solution. Use the following tables to dilute a set of standard stock solutions and construct an eight-point standard curve.

Table 1: Standard Stock Preparation

Standard	Testosterone Conc. (ng/mL)	Vol. of EIA Buffer (μL)	Transfer Vol. (μL)	Final Vol. (μL)
A	1000	-	Provided	80
B	20	980	20 μL of A	800
C	2	1800	200 μL of B	1800
D	0.2	1800	200 μL of C	2000

Table 2: Standard Curve Preparation

Standard	Testosterone Conc. (ng/mL)	Vol. of EIA Buffer (μL)	Vol. of Stock D (μL)
S ₀	0	1000	-
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	-	1000

ASSAY PROCEDURE

1. Add 50 μL of Standards or Samples to the corresponding wells on the microplate in duplicate. See **Scheme I** for a sample plate layout.
2. Add 50 μL of diluted Testosterone-HRP Conjugate to each well. Incubate at room temperature for one hour.
3. Wash the plate three times with 300 μL of diluted Wash Buffer per well. Wash 5 times if using an automated plate washer.
4. Add 150 μL of TMB Substrate to each well. Incubate at room temperature for 30 minutes.
5. Read the plate at 650 nm.

Alternately, the color reaction can be stopped after 10-15 minutes by adding 50 μL of 1 N HCl and read at 450 nm.

NOTE: If accounting for substrate background, use 2 wells as blanks (BLK) with only 150 μL TMB Substrate in the wells. Subtract the average of these absorbance values from the absorbance values of the wells being assayed.

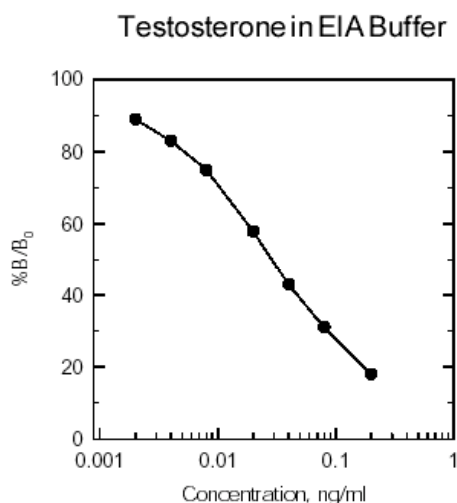
Scheme I: Sample Plate Layout

	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	BLK	BLK

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 y-axis against concentration on the 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.

Figure 1: Typical Standard Curve



CROSS REACTIVITY

Testosterone	100.0%	Testosterone 17 β -Cypionate	0.02%
Dihydrotestosterone	100.0%	Aldosterone	<0.01%
Androstenedione	0.86%	Corticosterone	<0.01%
Testosterone Enanthate	0.13%	Cortisol	<0.01%
Estriol	0.10%	Cortisone	<0.01%
Testosterone Benzoate	0.10%	Estrone	<0.01%
Estradiol	0.05%	17-Hydroxyprogesterone	<0.01%
Dehydroepiandrosterone	0.04%	Pregnenolone	<0.01%
Testosterone Propionate	0.04%	Progesterone	<0.01%
Deoxycorticosterone	0.03%		

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Oxford Biomedical Research, Inc.
P.O. Box 522
Oxford, MI 48371 U.S.A.

Orders: 800-692-4633
Technical Service: 248-852-8815
Fax: 248-852-4466
E-mail: info@oxfordbiomed.com

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