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TESTOSTERONE

ELISA KIT INSTRUCTIONS

PRODUCT # EA 78

PLEASE READ ALL INSTRUCTIONS CAREFULLY BEFORE BEGINNING THIS ASSAY

*****Store kit at 4° C at all times*****

CAUTION
This product is sold for research and/or in vitro use only. Not for clinical diagnostic use.

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.

MATERIALS

1. **EIA BUFFER:** 30 mL. To be used to dilute enzyme conjugate and Testosterone standards.
2. **WASH BUFFER 10x:** 20 mL. To be diluted 10x with deionized water. This is used to wash all unbound enzyme conjugate, samples and standards from the plate after the one hour incubation.
3. **SUBSTRATE:** 20 mL. Stabilized 3,3', 5,5' Tetramethylbenzidine (TMB) plus Hydrogen Peroxide (H2O2) in a single bottle. It is used to develop the color in the wells after they have been washed.
4. **EXTRACTION BUFFER 5x:** 30 mL. To be diluted 5x with deionized water. This is used for diluting extracted and non-extracted samples.
5. **TESTOSTERONE ENZYME CONJUGATE:** 150 mL. Testosterone horseradish peroxidase concentrate. Blue capped vial.
6. **TESTOSTERONE STANDARD:**100 mL. Testosterone standard at the concentration of 1 mg/mL. Green capped vial.
7. **TESTOSTERONE ANTIBODY CAOTED PLATE:** A 96 WELL MaxiSorp ™ Nunc microplate with antiTestosterone 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 for diluting 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. Plastic film or plate cover to cover plate during incubation.

OPTIONAL MATERIALS

7. 1 M HCl or Manufacturer's Stop Solution.
8. Microplate shaker.

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

9. Ethyl ether
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 your tip is unclean you could contaminate your 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 pouch sealed when not in use to maintain a dry environment. Seal with a
   heat sealer. If a heat sealer is not available, thoroughly close the open end with tape. Try to remove excess air before sealing.
3. Always use different pipette tips for the 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 cause cross contamination.
6. Standards and samples should be assayed in duplicate.

7. To quantitate, always run a standard curve when testing samples. If testing a sample that is not extracted, dilute standards in the same type of medium being tested, which is 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 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

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 mutliplied 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 5x 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 (this is provided)

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

E take 200 µL of D, add to 1.8 mL of EIA buffer and mix = 0.02 ng/mL

Continue standard preparation following Scheme I.

Scheme I

Standard	ng/mL	EIA buffer (µL added)	C Standard µL	D Standard µL	E Standard µL
S_0	0	as is	-	-	-
S_1	0.002	900	-	-	100
S_2	0.004	800	-	-	200
S_3	0.008	600	-	-	400
S_4	0.02	-	-	-	as is
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.

NOTE: Allow for extra wells when calculating amount of conjugate to dilute to allow for loss during pipetting (i.e. 4 extra wells if using a single pipette; 10 extra wells if using a multichannel pipette).

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.
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 washing buffer. Repeat for a total of three washings. (An automated plate washer can be used.)
11. Add 150 µL of substrate to each well. (Use multichannel pipette for best results.) Mix by shaking plate gently.
12. Allow to stand at room temperature for 30 minutes.
13. Gently shake plate before taking a reading to insure 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 M HCl or Manufacturer's Stop Solution to each well to stop enzyme reaction.

17. Read plate at 450 nm,if 1 M HCl solution was used. Read plate at 650 nm, if Manufacturer's 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

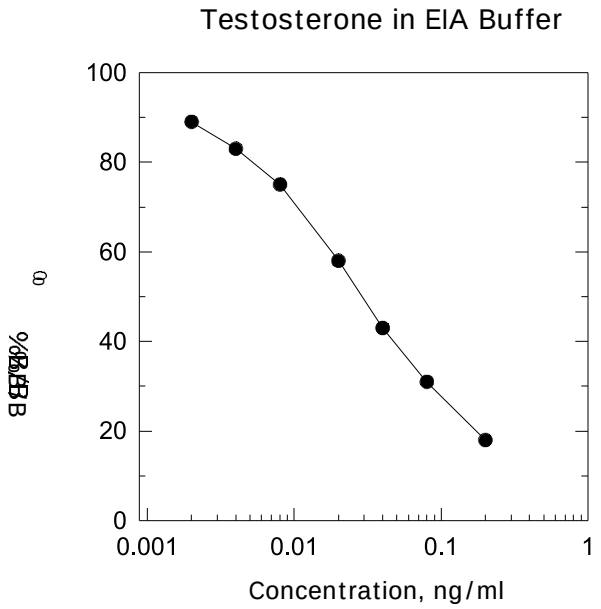
	1	2	3	4	5	6	7	8	9	10	11	12
A	s0	s0	u1	u1	u9	u9	u17	u17	u25	u25	u33	u33
B	s1	s1	u2	u2	u10	u10	u18	u18	u26	u26	u34	u34
C	s2	s2	u3	u3	u11	u11	u19	u19	u27	u27	u35	u35
D	s3	s3	u4	u4	u12	u12	u20	u20	u28	u28	u36	u36
E	s4	s4	u5	u5	u13	u13	u21	u21	u29	u29	u37	u37
F	s5	s5	u6	u6	u14	u14	u22	u22	u30	u30	u38	u38
G	s6	s6	u7	u7	u15	u15	u23	u23	u31	u31	u39	u39
H	s7	s7	u8	u8	u16	u16	u24	u24	u32	u32	u40	u40

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 $PGF_{2\alpha}$ standard.
7. If the samples were diluted, the concentration determined from the standard curve must be multiplied by the dilution factor.

TYPICAL STANDARD CURVE



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	100.00%
DIHYDROTESTOSTERONE	100.00%
ANDROSTENEDIONE	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%

HANDLING & STORAGE INSTRUCTIONS

Safety glasses and gloves should be worn to prevent skin and eye contact. Wear protective clothing such as lab coats to prevent contact. Store kit at 4°C.

PRECAUTIONS

1. Do not pipette solutions by mouth.
2. Reagents may contain sodium azide. Sodium azide may react with lead and copper plumbing to form explosive metal azides. On disposal of reagents, flush with large volumes of water to prevent azide accumulation.
3. Use only the 96-well PRECOATED plate supplied with the kit.
4. Do not eat or smoke in areas where specimens or kit reagents are being handled.

MATERIAL SAFETY DATA SHEET

Gloves and lab coat should be worn at all times while performing this assay. Contents may be harmful if swallowed, inhaled or absorbed through the skin. See *Precautions for Use*.

PHYSICAL AND CHEMICAL DATA

Components are stable in closed containers under normal temperatures and pressures.

HEALTH HAZARDS

Individual components may cause skin irritation or be harmful if swallowed. Avoid contact with skin and eyes.

FIRST AID

Call a physician. If swallowed give water or milk to dilute and induce vomiting. In case of contact with eyes, flush with copious amounts of water for at least 15 minutes. Assure adequate flushing by separating eyelids with fingers. In case of skin contact, wash with soap or mild detergent and large amounts of water.

DISCLAIMER

This information is believed to be correct but does not purport to be all inclusive and shall be used only as a guide. Oxford Biomedical Research, Inc. shall not be held liable for any damage resulting from handling or from contact with the above product. See catalog for additional terms and conditions of sale.

ORDERING INFORMATION

For additional kits or a complete catalog please call 1-800-692-4633.

TECHNICAL SUPPORT

If you need technical information or assistance with assay procedures, please call our Technical Support Department at 1-800-692-4633 or 1-248-852-8815. Our staff will answer your questions about this or any other product in the Oxford Biomedical line.

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