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LDN[®]

Instructions for use

Free Testosterone ELISA

REF

AA E-1400



IVD



Free Testosterone ELISA

INTENDED USE

For the direct quantitative determination of Free Testosterone by enzyme immunoassay in human serum.
For *in vitro* diagnostic use only.

PRINCIPLE OF THE TEST

The principle of the following enzyme immunoassay test follows the typical competitive binding scenario. Competition occurs between an unlabeled antigen (present in standards, controls and patient samples) and an enzyme-labelled antigen (conjugate) for a limited number of antibody binding sites on the microwell plate. The washing and decanting procedures remove unbound materials. After the washing step, the enzyme substrate is added. The enzymatic reaction is terminated by addition of the stopping solution. The absorbance is measured on a microtiter plate reader. The intensity of the colour formed is inversely proportional to the concentration of free testosterone in the sample. A set of standards is used to plot a standard curve from which the amount of free testosterone in patient samples and controls can be directly read.

The free testosterone kit utilizes a highly specific rabbit anti-testosterone polyclonal antibody at a low binding capacity ($K_{eq} \times$ concentration) to keep minimum disturbances of the testosterone-protein equilibrium. The other components in the test system are also optimized in order to not alter the original free testosterone concentration.

CLINICAL APPLICATIONS

Testosterone is a C-19 steroid secreted from the testis and the adrenal cortex in men and from the adrenal cortex and ovaries in women. Testosterone is also produced by peripheral tissues from androstenedione, which is of little physiological significance in men, however in women about half of the circulating testosterone is derived from this origin. Testosterone measurements are used mainly for clinical evaluation of hypogonadism in males and hyperandrogenic states in females.

Testosterone circulates in the blood bound to three proteins: sex hormone binding globulin (60-80%), albumin and cortisol binding globulin. Only about 1-2% of the total circulating testosterone remains unbound or free. Even though it is still under investigation, most researchers accept the free testosterone determination as a measure of the biologically active fraction. Free testosterone determinations are recommended to overcome the influences caused by variations in transport proteins on the total testosterone concentration.

PROCEDURAL CAUTIONS AND WARNINGS

1. Users should have a thorough understanding of this protocol for the successful use of this kit. Reliable performance will only be attained by strict and careful adherence to the instructions provided.
2. Control materials or serum pools should be included in every run at a high and low level for assessing the reliability of results.
3. When the use of water is specified for dilution or reconstitution, use deionized or distilled water.
4. In order to reduce exposure to potentially harmful substances, gloves should be worn when handling kit reagents and human specimens.
5. All kit reagents and specimens should be brought to room temperature and mixed gently but thoroughly before use. Avoid repeated freezing and thawing of reagents and specimens.
6. A calibrator curve must be established for every run.
7. The controls should be included in every run and fall within established confidence limits.
8. Improper procedural techniques, imprecise pipetting, incomplete washing as well as improper reagent storage may be indicated when assay values for the controls do not reflect established ranges.
9. When reading the microplate, the presence of bubbles in the microwells will affect the optical densities (ODs). Carefully remove any bubbles before performing the reading step.
10. The substrate solution (TMB) is sensitive to light and should remain colourless if properly stored. Instability or contamination may be indicated by the development of a blue colour, in which case it should not be used.
11. When dispensing the substrate and stopping solution, do not use pipettes in which these liquids will come into contact with any metal parts.
12. To prevent contamination of reagents, use a new disposable pipette tip for dispensing each reagent, sample, standard and control.
13. Do not mix various lot numbers of kit components within a test and do not use any component beyond the expiration date printed on the label.
14. Kit reagents must be regarded as hazardous waste and disposed of according to national regulations.

LIMITATIONS

1. All the reagents within the kit are calibrated for the direct determination of free testosterone in human serum. The kit is not calibrated for the determination of free testosterone in other specimens of human or animal origin.
2. Do not use grossly hemolyzed, grossly lipemic, icteric or improperly stored serum.
3. Any samples or control sera containing azide or thimerosal are not compatible with this kit, as they may lead to false results.
4. Samples reading higher than 100 pg/ml should not be diluted. Dilution will alter the equilibrium between free testosterone and serum proteins.
5. The results obtained with this kit should never be used as the sole basis for a clinical diagnosis. For example, the occurrence of heterophilic antibodies in patients regularly exposed to animals or animal products has the potential of causing interferences in immunological tests. Consequently, the clinical diagnosis should include all aspects of a patient's background including the frequency of exposure to animals/products if false results are suspected.

SAFETY CAUTIONS AND WARNINGS

POTENTIAL BIOHAZARDOUS MATERIAL

Human serum that may be used in the preparation of the standards and controls has been tested and found to be non-reactive for Hepatitis B surface antigen and has also been tested for the presence of antibodies to HCV and Human Immunodeficiency Virus (HIV) and found to be negative. However no test method can offer complete assurance that HIV, HCV and Hepatitis B virus or any infectious agents are absent. The reagents should be considered a potential biohazard and handled with the same precautions as applied to any blood specimen.

CHEMICAL HAZARDS

Avoid contact with reagents containing TMB, hydrogen peroxide and sulfuric acid. If contacted with any of these reagents, wash with plenty of water. TMB is a suspected carcinogen.

SPECIMEN COLLECTION AND STORAGE

Approximately 0.1 ml of serum is required per duplicate determination. Collect 4-5 ml of blood into an appropriately labelled tube and allow it to clot. Centrifuge and carefully remove the serum layer. Store at 4°C for up to 24 hours or at -10°C or lower if the analyses are to be done at a later date. Consider all human specimens as possible biohazardous materials and take appropriate precautions when handling.

SPECIMEN PRETREATMENT

This assay is a direct system; no specimen pretreatment is necessary.

REAGENTS AND EQUIPMENT NEEDED BUT NOT PROVIDED

- Precision pipettes to dispense 25, 50, 100, 150 and 300 µl
- Disposable pipette tips
- Distilled or deionized water
- A 37 °C incubator
- Microwell plate reader with a filter set at 450nm and an upper OD limit of 3.0 or greater* (see assay procedure step 11).

REAGENTS PROVIDED

AA E-0030 WASH-CONC 10x Wash Buffer Concentrate – X10

Contents: One bottle containing buffer with a non-ionic detergent and a non-mercury preservative.
Volume: 50 ml/bottle
Storage: Refrigerate at 2-8°C
Stability: 12 months or as indicated on label.
Preparation: Dilute 1:10 in distilled or deionized water before use. If the whole plate is to be used dilute 50 ml of the wash buffer concentrate in 450 ml of water.

AA E-0055 SUBSTRATE TMB Substrate - Ready To Use.

Contents: One bottle containing tetramethylbenzidine and hydrogen peroxide in a non-DMF or DMSO containing buffer.
Volume: 16 ml/bottle
Storage: Refrigerate at 2-8°C
Stability: 12 months or as indicated on label.

AA E-0080 **STOP-SOLN** **Stopping Solution** - Ready To Use.

Contents: One vial containing 1M sulfuric acid.
 Volume: 6 ml/vial
 Storage: Refrigerate at 2-8°C
 Stability: 12 months or as indicated on label.

Calibrators and **Controls**- Ready To Use.

Listed below are approximate concentrations, please refer to vial labels for exact concentrations:

Cat. no.	Symbol	Calibrator	Concentration	Volume/Vial
AA E-1401	STANDARD A	Calibrator A	0 pg/ml	0.5 ml
AA E-1402	STANDARD B	Calibrator B	0.25 pg/ml	0.5 ml
AA E-1403	STANDARD C	Calibrator C	1.02 pg/ml	0.5 ml
AA E-1404	STANDARD D	Calibrator D	5.5 pg/ml	0.5 ml
AA E-1405	STANDARD E	Calibrator E	25 pg/ml	0.5 ml
AA E-1406	STANDARD F	Calibrator F	125 pg/ml	0.5 ml
AA E-1451	CONTROL 1	Control 1	Refer to vial labels for expected value and acceptable range!	0.5 ml
AA E-1452	CONTROL 2	Control 2		0.5 ml

Contents: Prepared by spiking serum with a precise quantity of testosterone equivalent to approximately 0, 0.25, 1.02, 5.5, 25 and 125 pg/ml of free testosterone.
 Storage: Refrigerate at 2-8°C
 Stability: 12 months in unopened vials or as indicated on label. Once opened, the standards should be used within 14 days or aliquoted and stored frozen. Avoid multiple freezing and thawing cycles.

AA E-1413 **ASSAY-BUFF** **Assay Buffer** - Ready To Use.

Contents: One vial containing a protein-based buffer with a non-mercury preservative.
 Volume: 15 ml/vial
 Storage: Refrigerate at 2-8°C
 Stability: 12 months or as indicated on label.

AA E-1431 **96** **Rabbit Anti-Free Testosterone Antibody Coated Microwell Plate-Break Apart Wells** - Ready To Use.

Contents: One 96 well (12x8) polyclonal antibody-coated microwell plate in a resealable pouch with desiccant.
 Storage: Refrigerate at 2-8°C
 Stability: 12 months or as indicated on label.


AA E-1440 **CONJUGATE-CONC 50x** **Free Testosterone-Horseradish Peroxidase (HRP) Conjugate Concentrate – X50**

Contents: Free Testosterone-HRP conjugate in a protein-based buffer with a non-mercury preservative.
 Volume: 300 µl/vial
 Storage: Refrigerate at 2-8°C
 Stability: 12 months or as indicated on label.
 Preparation: Dilute 1:50 in assay buffer before use (eg. 40 µl of HRP in 2 ml of assay buffer). If the whole plate is to be used dilute 240 µl of HRP in 12 ml of assay buffer. Discard any that is left over.

ASSAY PROCEDURE

Specimen Pretreatment: **None.**

All reagents must reach room temperature before use. Calibrators, controls and specimen samples should be assayed in duplicate. Once the procedure has been started, all steps should be completed without interruption.

1.	Prepare working solutions of the free testosterone-HRP conjugate and wash buffer .
2.	Remove the required number of microwell strips. Reseal the bag and return any unused strips to the refrigerator.
3.	Pipette 25 µl of each calibrator, control and specimen sample into correspondingly labelled wells in duplicate.
4.	Pipette 100 µl of the conjugate working solution into each well. (We recommend using a multichannel pipette).
5.	Gently shake the plate for 10 seconds .
6.	Incubate the plate at 37°C for 1 hour
7.	Wash the wells 3 times with 300 µl of diluted wash buffer per well and tap the plate firmly against absorbent paper to ensure that it is dry (The use of a washer is recommended).
8.	Pipette 150 µl of TMB substrate into each well at timed intervals.
9.	Incubate the plate at 37°C for 10-15 minutes . (or until Calibrator A attains dark blue colour for desired OD).
10.	Pipette 50 µl of stopping solution into each well at the same timed intervals as in step 8.
11.	Read the plate on a microwell plate reader at 450 nm within 20 minutes after addition of the stopping solution.
	<i>If the OD exceeds the upper limit of detection or if a 450nm filter is unavailable, a 405 or 415nm filter may be substituted. The optical densities will be lower, however, this will not affect the results of patient/control samples.</i>

CALCULATIONS

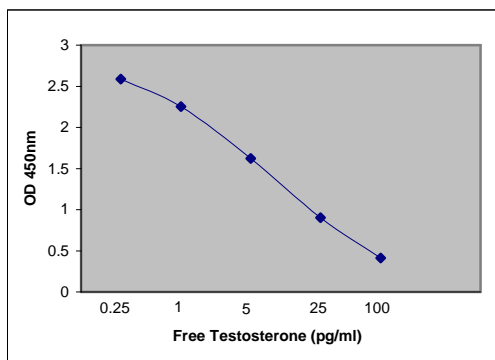
- Calculate the mean optical density of each calibrator duplicate.
- Draw a calibrator curve on semi-log paper with the mean optical densities on the Y-axis and the calibrator concentrations on the X-axis. If immunoassay software is being used, a 4-parameter or 5-parameter curve is recommended.
- Calculate the mean optical density of each unknown duplicate.
- Read the values of the unknowns directly off the calibrator curve.

TYPICAL TABULATED DATA:

Calibrator	OD 1	OD 2	Mean OD	Value (pg/ml)
A	2.100	2.013	2.057	0
B	1.463	1.506	1.485	0.25
C	0.908	0.922	0.915	1.02
D	0.472	0.462	0.467	5.5
E	0.277	0.254	0.266	25
F	0.153	0.146	0.150	125
Unknown	0.464	0.458	0.461	5.7

TYPICAL CALIBRATOR CURVE

Sample curve only. **Do not** use to calculate results:



PERFORMANCE CHARACTERISTICS

SENSITIVITY

The lower detection limit is calculated from the standard curve by determining the resulting concentration of the mean OD of Calibrator A (based on 10 replicate analyses) minus 2 SD. Therefore, the sensitivity of the Direct Free Testosterone ELISA kit is **0.17 pg/ml**.

SPECIFICITY (CROSS REACTIVITY)

The following compounds were tested for cross-reactivity with the Direct Free Testosterone ELISA kit with testosterone cross-reacting at 100%.

Steroid	%Cross Reactivity
Testosterone	100
5 α -DHT	5.2
Androstenedione	1.4
Androstanediol	0.8
Progesterone	0.5
Androsterone	0.1

The following steroids were tested but cross-reacted at less than 0.1%: Aldosterone, Andrenosterone, Cholesterol, Corticosterone, Dehydroepiandrosterone, Dehydroepiandrosterone Sulfate, Epiandrosterone, 17 β -Estradiol, Estriol and Pregnenolone.

INTRA-ASSAY PRECISION

Three samples were assayed ten times each on the same calibrator curve. The results (in pg/ml) are tabulated below:

Sample	Mean	SD	CV%
1	1.17	0.20	17.0
2	15.96	0.79	4.9
3	62.46	2.95	4.7

INTER-ASSAY PRECISION

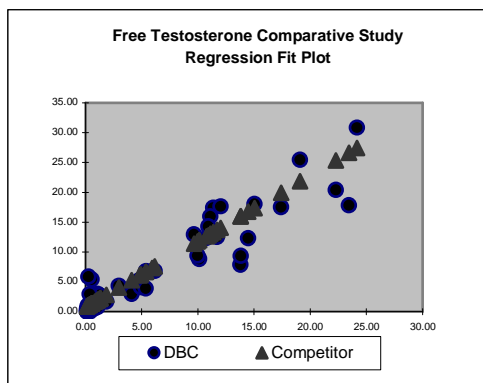
Three samples were assayed ten times over a period of two weeks. The results (in pg/ml) are tabulated below:

Sample	Mean	SD	CV%
1	0.97	0.12	12.4
2	25.81	1.36	5.3
3	75.81	6.66	8.8

COMPARATIVE STUDIES

The Direct Free Testosterone ELISA Kit (y) was compared with a competitors Free Testosterone Coated Tube RIA Kit (x). The comparison of 61 serum samples yielded the following linear regression results:

$$y = 1.0137x (\text{competitor}) + 0.6404$$
$$r = 0.89$$



EXPECTED NORMAL VALUES

As for all clinical assays each laboratory should collect data and establish their own range of expected normal values. The results of an expected range study with apparently normal healthy subjects yielded the following results (all values are reported in pg/ml):

Group	N	Median	Central 95% Range	Absolute Range
Males	71	12.3	4.25-30.37	3.84-34.17
Females	60	1.03	0.04-4.18	0.01-7.01

EFFECT OF SEX HORMONE BINDING GLOBULIN (SHBG)

The purpose of this study was to investigate a possible interference caused by the binding of SHBG to the free testosterone-horse radish peroxidase conjugate. A charcoal-stripped human serum pool was spiked precisely with SHBG at concentrations ranging from 6-200 µg/ml and was assayed with the Direct Free Testosterone ELISA Kit. Results tabulated below (in pg/ml):

SHBG Added	OD 450nm	Percent B/B ₀ (%)
0 µg/ml	2.34	100.0
6.25 µg/ml	2.33	99.7
12.5 µg/ml	2.27	97.2
50 µg/ml	2.14	91.6
200 µg/ml	2.10	89.7

The results showed bound values between 90-100% of B/B₀ (B₀=unspiked serum) even at higher than normal (0.5-5 µg/ml) SHBG levels. In conclusion, the results showed that there was no significant influence by SHBG in the Direct Free Testosterone Direct ELISA kit.

EFFECT OF HUMAN SERUM ALBUMIN (HSA)

The purpose of this study was to investigate a possible interference of human serum albumin (HSA) on the assay procedure. HSA was added to three patient samples at concentrations of 1.25, 2.5 and 5.0 g/dl. All samples were assayed with the Direct Free Testosterone ELISA Kit and yielded the following results (in pg/ml):













Sample	Added HSA g/dl			
	0	1.25	2.5	5.0
1	0.52	0.34	0.54	0.53
2	15.8	14.2	12.5	10.9
3	26.2	23.0	21.0	18.6

The results demonstrate no significant influence of added HSA on the three patient serum samples.

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Symbols:

	Storage temperature		Manufacturer		Contains sufficient for <n> tests
	Expiry date		Batch code		For in-vitro diagnostic use only!
	Consult instructions for use		Content		CE labelled
	Caution		Catalogue number		For research use only!