Labor Diagnostika Nord GmbH & Co. KG
Am Eichenhain 1, 48531 Nordhorn
Telefon: +49-5921-8197 0
Telefax: +49-5921-8197 222
e-mail: manz@ldn.de
Internet: http://www.ldn.de

Instructions for use

Free Testosterone ELISA

REF AA E-1400

96

+2 +8 °C

IVD

CE

Free Testosterone ELISA

1. Intended use and principle of the test

Enzyme Immunoassay for the direct quantitative determination of Free Testosterone in human serum

The principle of the following enzyme immunoassay test follows the typical competitive binding scenario. Competition occurs between an unlabeled antigen (present in standards, control 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 LDN free testosterone direct ELISA utilizes a highly specific rabbit anti-testosterone polyclonal antibody at a low binding capacity (Keq x 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.

2. Procedural cautions and warnings

This kit is intended for in vitro use only.

Practice the following good laboratory practices when handling kit reagents:

- Do not pipette by mouth.
- Do not smoke, drink, or eat in areas where specimens or kit reagents are being handled.
- Wear protective clothing and disposable gloves when handling the specimens and kit reagents.
- Wash hands thoroughly after performing the test.
- Avoid contact with eyes; use safety glasses; in case of contact, flush with water immediately and contact a doctor.
- 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.
- Avoid microbial contamination of reagents.
- A Standard curve must be established for every run.
- It is recommended to all customers to prepare their own control materials or serum pools which should be included in every run at a high and low level for assessing the reliability of results.
- The controls (included in kit) should be included in every run and fall within established confidence limits.
- When the use of water is specified for dilution or reconstitution, use deionized or distilled water.
- 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.
- Improper procedural techniques, imprecise pipetting, incomplete washing as well as improper reagent storage may be indicated when assay values for the control do not reflect established ranges.
- 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.
- 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.
- When dispensing the substrate and stopping solution, do not use pipettes in which these liquids will come into contact with any metal parts.
- To prevent contamination of reagents, use a new disposable pipette tip for dispensing each reagent, sample, standard and control.
- 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.
- Kit reagents must be regarded as hazardous waste and disposed of according to local and/or national regulations.

3. Limitations

- Do not use grossly hemolyzed, grossly lipemic, icteric or improperly stored serum.
- Any samples or control sera containing azide or thimerosal are not compatible with this kit, as they may lead to false results.
- Only assay buffer may be used to dilute any high serum samples. The use of any other reagent may lead to false results.
- 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.

4. Safety cautions and warnings

POTENTIAL BIOHAZARDOUS MATERIAL

All serum samples should be considered a potential biohazard and handled with the appropriate precautions.

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.

5. Sample 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.

6. Additional materials and equipment required but not provided in the kit

- 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 test procedure step 11).

7. Reagents provided

The Free Testosterone ELISA kit AA E-1400 contains enough reagents for 96 quantitative determinations.

96 **AA E-1431 Rabbit Anti-Free Testosterone Antibody Coated Microwell Plate**

- Ready To Use
Break Apart Wells.
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.

CONJUGATE-CONC 50x **AA E-1440 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 12ml of assay buffer. Discard any that is left over.

Free Testosterone Standards - Ready To Use
Contents: Six vials containing testosterone in a human serum-based buffer with a non-mercury preservative. 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.
*Listed below are approximate concentrations, please refer to vial labels for exact concentrations.

	Catalogue no.	Standard	Concentration	Volume/Vial
STANDARD A	**AA E-1401**	Standard A	0 pg/ml	0.5 ml
STANDARD B	**AA E-1402**	Standard B	0.25 pg/ml	0.5 ml
STANDARD C	**AA E-1403**	Standard C	1.02 pg/ml	0.5 ml
STANDARD D	**AA E-1404**	Standard D	5.5 pg/ml	0.5 ml
STANDARD E	**AA E-1405**	Standard E	25 pg/ml	0.5 ml
STANDARD F	**AA E-1406**	Standard F	125 pg/ml	0.5 ml

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.

CONTROL 1 **AA E-1451 Control 1** - Ready To Use

CONTROL 2 **AA E-1452 Control 2** - Ready To Use

Contents: Two vials containing testosterone in a human serum-based buffer with a non-mercury preservative. Prepared by spiking serum with a precise quantity of testosterone. Refer to vial label for expected value and acceptable range.
Volume: 0.5 ml/vial
Storage: Refrigerate at 2-8°C
Stability: 12 months in unopened vial or as indicated on label. Once opened, the control should be used within 14 days or aliquoted and stored frozen. Avoid multiple freezing and thawing cycles.

WASH-CONC 10x **AA E-0030 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.

ASSAY-BUFF **AA E-1413 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.

SUBSTRATE **AA E-0055 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.

STOP-SOLN **AA E-0080 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.

8. Test procedure

All reagents must reach room temperature before use. Standards, 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 standard, control and serum sample into the corresponding wells in duplicate.
4.	Pipette **100 µL** of the conjugate working solution into each well.
5.	Gently shake the plate for **10 seconds**.
6.	Incubate the plate for **1 hour** at **37 °C**.
7.	Wash the wells **3 times** with prepared wash buffer (300 µL/well for each wash) 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 Standard 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.

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.

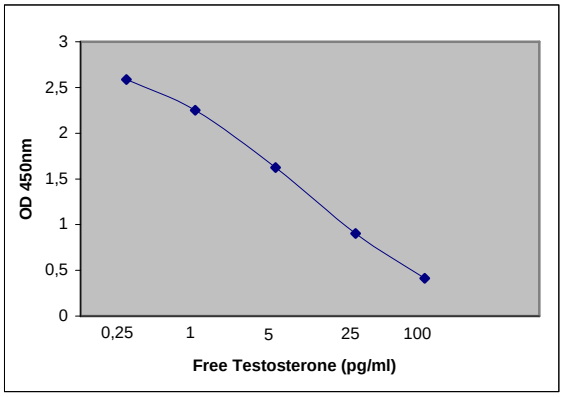
9. Calculation of results

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

Typical tabulated data (example, do not use for your own calculation)

Standard	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 Standard curve (example, do not use for your own calculation)



10. Assay characteristics

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

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

Steroid	% Cross Reactivity
Testosterone	100
5α-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, 17β-Estradiol, Estriol and Pregnenolone.

Intra-assay precision

Three samples were assayed ten times each on the same Standard 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 study

The 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 (LDN)=1.0137x (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 Free Testosterone ELISA Kit. Results tabulated below (in pg/ml):

SHBG Added	OD 450nm	Percent B/B_o (%)
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/Bo (Bo=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 LDN 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 dbc 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.

Actual literature, information about clinical significance or any other information about the test are available on the homepage or contact the manufacturer directly.

Symbols:

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