

**REF**

GD7282 00

FREE TESTOSTERONE

Enzyme-immunoassay for the quantitative determination of Free Testosterone in serum or plasma

**IVD**

INDICATION

Testosterone is a steroid hormone from the androgen group. Testosterone is primarily secreted in the testes of males and the ovaries of females although small amounts are secreted by the adrenal glands. It is the principal male sex hormone and an anabolic steroid. In both males and females, it plays key roles in health and well-being.

Only 1-2% of circulating testosterone exists as unbound or free testosterone. The majority, approximately 60%, is bound to SHBG with high affinity, while the remainder is loosely bound to albumin. Both the albumin-bound and free fractions may be biologically active, while SHBG effectively inhibits testosterone action. Testosterone effects can be classified as virilising and anabolic effects. Anabolic effects include growth of muscle and bone mass and strength. Virilizing effects include maturation of the sex organs. In men testosterone levels decline gradually with age.

Measurement of the free or unbound fraction of serum testosterone has been proposed as a means of estimating the physiologically bioactive hormone. Free testosterone levels are elevated in women with hyperandrogenism associated with hirsutism in the presence or absence of polycystic ovarian disease. In addition, free testosterone measurements may be more useful than total testosterone in situations where SHBG is increased or decreased (e.g. hypothyroidism and obesity).

PRINCIPLE OF THE ASSAY

This test is based on "one step" competition enzyme immunoassay principle (ELISA). Tested specimen is placed into the microwells coated by specific anti-Testosterone antibodies simultaneously with Testosterone conjugated to Horseradish peroxidase (HRP). Free testosterone from the specimen competes with the conjugated antigen for coated antibodies. After washing procedure, the remaining enzymatic activity bound to the microwell surface is detected and quantified by addition of chromogen-substrate solution. The developed colour, detected at 450 nm, is inversely related to the quantity of Free Testosterone present in the specimen.

Free Testosterone concentration in the sample is calculated based on a series of standards.

KIT CONTENT

1. Reagent A – Microplate

12x8 strips.

8 wells breakable strips, coated with anti-Testosterone antibody. The strips are assembled on a plastic frame and contained in a sealed bag with desiccant. Bring the strips to room temperature before use, to prevent any moisture formation inside the bag.

2. Reagent B – Enzymatic Tracer

1 vial of 15 ml.

Testosterone, conjugated with Horseradish peroxidase (HRP).

3. Reagent C – Washing Solution 10x

1 vial of 50 ml.

Concentrated solution to be diluted 1:10 with distilled water.

It contains Phosphate buffer 0.2 M, Proclin 0.002%.

4. Reagent D/E – Chromogen/Substrate

1 vial of 15 ml.

Ready to use solution containing Tetramethylbenzidine (TMB) 0.25 g/l.

Avoid any skin contact and light exposure.

5. Reagent F – Stop Solution

1 vial of 15 ml.

Ready to use solution containing Sulphuric acid 0.15 M.

Avoid any skin contact.

6. Free Testosterone Standards:

6 vials of 1 ml each.

Ready to use liquids containing Free Testosterone approximately at the following concentrations:

S₀: 0 pg/ml, **S₁:** 0.2 pg/ml, **S₂:** 1 pg/ml, **S₃:** 4 pg/ml, **S₄:** 20 pg/ml, **S₅:** 100 pg/ml.

Actual concentrations to be used for calculation are stated on the labels of the vials.

7. Cardboard sealers:

2 cardboard sealers to be used to cover the plate during the incubations.

8. Package insert: instruction for use GD7282 00 it/ing.

MICROBIOLOGICAL STATE AND CLEANING GRADE

1. All the materials of human origin resulted negative to HbsAg, HIV 1&2 and HCV FDA approved tests. Anyhow, as no test can guarantee the absolute absence of infective agents, handle reagents as potentially infected, especially standards, controls and samples. All objects come in direct contact with samples and all residuals of the assay should be treated or eliminated as potentially infected. Best procedures for inactivation are treatments with autoclave at 121°C for 30 minutes or with sodium hypochlorite at a final concentration of 2.5 % for 24 hours.
2. Avoid any contact with skin and mucous membrane, in particular for Stop Solution.
3. Use protective disposable talk-free gloves.
4. Avoid contaminating reagents when taking them from the vials. We recommend to use automatic pipettes with disposable tips. When dispensing reagents, do not touch with tips the wall of wells in order to avoid cross-contaminations.
5. For the washing step, follow carefully the indications reported in "WASHING INSTRUCTION".
6. Avoid the substrate/chromogen to come in contact with oxidizing agents or metallic surfaces; avoid intense light exposure during incubation or reagent preparation.

STORAGE AND STABILITY OF THE KIT

1. The kit has to be stored at 2-8 °C and used before the expiry date stated on the label.
2. Unused strips have to be placed in the bag containing the desiccant and firmly sealed before restore at 2-8 °C. After opening the strips are stable up to the expiry date stated on the label.
3. All other reagents can be repeatedly used up to exhaustion if stored at 2-8 °C, provided that they are handled carefully to avoid any environment contamination. Under these conditions the reagents are stable up to the expiry date stated on the labels.

AUXILIARY MATERIALS

- Semi automatic pipettes of 10, 200 and 1000 µl
- Vortex mixer and absorbent paper
- Chronometer
- Ultrapure Elisa grade water
- Microplate incubator at 37 ± 1 °C.
- Photometric reader of microplates or microstrips, linear up to at least 2 OD and supplied with filter of 450 nm (620- 630 nm).
- Automatic microplates washing device or manual apparatus capable of aspirating and dispensing volumes of 300 µl.

SAMPLES

Serum or plasma (heparin, EDTA). Samples can be stored at 2-8 °C for a short time (max 1 day). For longer storage the specimen should be frozen. Avoid repeated freezing and thawing. Highly lipemic, hemolysed or microbiologically contaminated samples should not be used in the assay.

REAGENTS PREPARATION

- **WASHING SOLUTION:** dilute 1:10 with distilled or ELISA grade water (ex.: 20 ml of reagent C + 200 ml of distilled water) and mix carefully before use. The diluted washing solution can be stored for one week at room temperature or four weeks at +2-8°C. It is recommended to store diluted washing solution at room temperature for immediate use. In Reagent C it is possible to observe the presence of crystals, in this case mix at room temperature until complete dissolution of crystals.

WASHING INSTRUCTION

A good washing procedure is essential to obtain correct and precise analytical results.

We therefore recommend to use a good quality ELISA microplate washer, maintained at a good level of washing mechanical performances.

Generally, 2-3 automatic washing cycles of 0.3 ml/well are sufficient to avoid false positive reactions and remove high background. Anyhow we recommend to calibrate the washing system on the kit itself so to match the declared analytical performances.

In case of manual washing, we suggest to perform 3 washing cycles, dispensing and aspirating 0.3 ml/well per cycle.

In any case the liquid washed out from the plates must be inactivated with a sodium hypochlorite solution at a final concentration of 2.5%, before being thrown away or autoclaved, as it must be considered as potentially infected.

ASSAY PROCEDURE

1. At least one hour before use, bring all reagents, standards and samples to room temperature (18-30°C), mixing them carefully on vortex.
2. Do not mix reagents from different lots.
3. We recommend to distribute standards and samples in duplicate.
4. Distribution and incubation times must be the same for all wells in the same analysis.
5. Avoid long interruptions between each step of the assay procedure.
6. It is suggested to eliminate the excess of washing solution from the microplate after washing by blotting it gently on an absorbent paper pad.
7. The colour developed in the last incubation is stable for a maximum of one hour. Otherwise, in case of reading after 10-15 min after dispensing stop solution, immediately place the strips **in the dark**.
8. We recommend to read the plate with an ELISA automatic reader able to subtract the background at 620-630 nm and to read the absorbance of samples and standards at 450 nm. The "blanking" of the instrument is to be carried out in the blank reagent well (well A1).

ASSAY SCHEME

- Put the desired number of microstrips into the frame.
- Follow the scheme:

	Microplate wells coated with anti-Testosterone antibody			
	REAGENTS	Blank	Standard	Sample
Immunological reaction	Standard	-	20 µl	-
	Sample	-	-	20 µl
	Reagent B (Enzymatic Tracer)	-	100 µl	100 µl
	- Cover the strips with cardboard sealer - Incubate 60 minutes at 37 ± 1 °C			
Washing	- Peel out the cardboard sealer and aspirate the reaction solution from all wells - Rinse 3 times with 300 µl of diluted washing solution, carefully aspirating off the remaining liquid			
Colorimetric reaction	Reagent D/E (Chromogen-Substrate)	100 µl	100 µl	100 µl
	- Cover the strips with cardboard sealer - Incubate 15 minutes at room temperature (22-28 °C) , avoiding light exposure			
	Reagent F (Stop Solution)	100 µl	100 µl	100 µl
	Read the absorbance of each well against Blank at 450 nm (and 620-630 nm)			

QUALITY CONTROL

It is recommended, in each analytical run, to use control sera with known free testosterone values, to check the correspondence of the obtained results with those expected and consequently validate the data.

CALCULATION OF RESULTS

- Calculate the mean of the absorbance (Em) for each point of the standard curve (S₀ – S₅) and of each sample.
- Plot the mean value of absorbance of the standards (Em) against proper Free Testosterone concentrations. Draw the best-fit curve through the plotted points. (ex.: Four Parameter Logistic).
- Interpolate the values of the samples on the standard curve to obtain the corresponding values of the concentrations expressed in pg/ml.
- If computer controlled data reduction is used to calculate the results of the test, it is imperative that the predicted values for the standards fall within 10% of the assigned concentrations.

EXPECTED VALUES

From data obtained by Minias Globe Diagnostics the following reference ranges are suggested. Otherwise, it is recommended that each laboratory establishes its own reference range.

Subjects	Ranges (pg/ml)
Male:	4.5-42.0
Female:	
ovulation	< 4.1
oral contraceptives	0.3-2.0
postmenopausal	0.1-1.7

Note:

The clinical significance of free testosterone determination can be invalidated if the patient was treated with cortisone or natural or synthetic steroids.

ANALYTICAL PERFORMANCES

Sensitivity

The lowest detectable concentration of Free Testosterone calculated from average absorbances of Standard 0 minus 2 Standard Deviation resulted 0.06 pg/ml.

Precision

a. Intra Assay Variation

Within run variation was determined by 15 replicate determinations of three different serum samples in the same analytical run. %CV values found were < 10%.

b. Inter Assay Variation

Between run variation was determined by replicate measurements of three different control sera and two serum samples in 10 different analytical runs. %CV values found were < 10%.

Correlation with RIA

The present kit was compared to a well-established RIA method. 24 female sera and 17 male sera were assayed with both methods.

The following linear regression curve was calculated:

$$y = 0.957x + 0.953 \text{ pg/ml} \quad r = 0.968$$

Specificity

Potentially cross reactive analytes and interfering substances (anticoagulants) have been tested. The cross reaction of the antibody calculated at 50% according to Abraham are the following:

Testosterone	100%
DHT	0.00008%
DHEA-S	0.00007%
Androstenedione	0.0043%
Androsterone	0.0029%
17 α Ethylenestradiol	< 0.00001%
17 β Estradiol	0.00005%
Estrone	< 0.00001%
Prednisone	< 0.00001%
Cortisone	< 0.00001%
Cortisol	< 0.00001%
Norgestrel	0.00001%
Danazolo	< 0.00001%
Aldosterone	< 0.00001%
Sodium Citrate	< 0.00001%
EDTA	< 0.00001%
Heparin	< 0.00001%

PRECAUTIONS IN USE

The reagents contain inactive components such as preservatives (Sodium azide or others), surfactants etc. The total concentrations of these components is lower than the limits reported by 67/548/EEC and 88/379/EEC directives about classification, packaging and labelling of dangerous substances. However, the reagents should be handled with caution, avoiding swallowing and contact with skin, eyes and mucous membranes. The use of laboratory reagents according to good laboratory practice is recommended.

Waste Management

Please refer to local legal requirements.

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