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FREE TESTOSTERONE ELISA

for routine analysis

Direct immunoenzymatic determination of Free Testosterone in human serum or plasma.

IVD | LOT See external label | 2°C 8°C | Σ = 96 test | REF DKO015

INTENDED USE

Eagle Biosciences Free Testosterone ELISA Assay Kit is a competitive immunoenzymatic colorimetric method for quantitative determination of Free Testosterone concentration in human serum or plasma.
Free Testosterone kit is intended for laboratory use only.

1. CLINICAL SIGNIFICANCE

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.
Due to its insolubility in aqueous solutions, for the most part Testosterone circulates in the blood bound to transport proteins. Only a small percentage (< 1%) 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 *virilizing* and *anabolic* effects. Anabolic effects include growth of muscle mass and strength, increased bone density and strength, and stimulation of linear growth and bone maturation. Virilizing effects include maturation of the sex organs.
Testosterone levels decline gradually with age in men.
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).

2. PRINCIPLE

Testosterone in the blood is bound to SHBG (60%) and in lower quantity to other proteins (for example albumin). Only the measurement of Free Testosterone (< 1% of Total Testosterone) permits the estimating of the hormone biologically active.
In this Free Testosterone ELISA Assay Kit, the (antigen) in the sample competes with the antigenic Testosterone conjugated with horseradish peroxidase (HRP) present in the Conjugate for binding to the antibodies anti-Testosterone coated on the microplate (solid phase). The Testosterone bound to proteins does not take part to this reaction, so it is washed away during the washing step (for total Testosterone measurement the ELISA Eagle Biosciences “Testosterone” kit is available).
After incubation, the bound/free separation is performed by a simple solid-phase washing.
Then the enzyme HRP in the bound-fraction reacts with the Substrate (H_2O_2) and the TMB Substrate and develops a blu color that changes into yellow when the Stop Solution (H_2SO_4) is added.
The color intensity is inversely proportional to the Free Testosterone concentration in the sample.
Free Testosterone concentration in the sample is calculated through a calibration curve.

3. REAGENTS, MATERIALS AND INSTRUMENTATION

3.1. Reagents and materials supplied in the kit

1. Calibrators (6 vial, 1 mL each)

CAL0	REF **DCE002/1506-1**
CAL1	REF **DCE002/1507-1**
CAL2	REF **DCE002/1508-1**
CAL3	REF **DCE002/1509-1**
CAL4	REF **DCE002/1510-1**
CAL5	REF **DCE002/1511-1**

2. Control (1 vial, 1 mL)
Control concentration is lot-specific and is indicated on the Certificate of Analysis REF **DCE045/1503-0**

3. Conjugate (1 vial, 15 mL)
Testosterone conjugated with Horseradish peroxidase (HRP) REF **DCE002/1502-1**

4. Coated Microplate (1 breakable microplate)

Anti Testosterone antibody adsorbed on microplate
REF DCE002/1503-1

5. TMB Substrate (1 vial, 15 mL)
H_2O_2-TMB (0.26 g/L) *(avoid any skin contact)*
REF DCE004-0

6. Stop Solution (1 vial, 15 mL)
Sulphuric acid 0.15 M *(avoid any skin contact)*
REF DCE005-0

7. 10X Conc. Wash Solution (1 vial, 50 mL)
NaCl 160 g/L; Tween-20 10 g/L; 0.2M Phosphate buffer, pH 7.4 **REF DCE054-0**

3.2. Reagents necessary not supplied
Distilled water.

3.3. Auxiliary materials and instrumentation
Automatic dispenser.
Microplates reader (450 nm, 620-630 nm)

Note
Store all reagents at 2÷8°C in the dark.
Open the bag of reagent 4 (Coated Microplate) only when it is at room temperature and close it immediately after use. Once opened, the microplate is stable until the expiry date of the kit.

4. WARNINGS
- This Free Testosterone ELISA Assay Kit is intended for in vitro use by professional persons only. Not for internal or external use in Humans or Animals.
- Use appropriate personal protective equipment while working with the reagents provided.
- Follow Good Laboratory Practice (GLP) for handling blood products.
- Some reagents of the Free Testosterone ELISA Assay Kit contain small amounts of Proclin 300^R as preservative. Avoid the contact with skin or mucosa.
- The TMB Substrate contains an irritant, which may be harmful if inhaled, ingested or absorbed through the skin. To prevent injury, avoid inhalation, ingestion or contact with skin and eyes.
- The Stop Solution consists of a diluted sulphuric acid solution. Sulphuric acid is poisonous and corrosive and can be toxic if ingested. To prevent chemical burns, avoid contact with skin and eyes.
- Avoid the exposure of reagent TMB/H_2O_2 to directed sunlight, metals or oxidants. Do not freeze the solution.
- This Free Testosterone ELISA Assay Kit allows the determination of Free Testosterone from 0.06 pg/mL to 100.0 pg/mL.
- The clinical significance of Free Testosterone determination can be invalidated if the sample was treated with cortisone or natural or synthetic steroids.

5. PRECAUTIONS
- Please adhere strictly to the sequence of pipetting steps provided in this protocol. The performance data represented here were obtained using specific reagents listed in this Instruction For Use.
- All reagents of the Free Testosterone ELISA Assay Kit should be stored refrigerated at 2-8°C in their original container. Any exceptions are clearly indicated. The reagents are stable until the expiry date when stored and handled as indicated.
- Allow all Free Testosterone ELISA Assay Kit components and specimens to reach room temperature (22-28°C) and mix well prior to use.
- Do not interchange Free Testosterone ELISA Assay Kit components from different lots. The expiry dates printed on the labels of the box and of the vials must be observed. Do not use any kit component beyond their expiry date.
- If you use automated equipment, the user has the responsibility to make sure that the kit has been appropriately tested. To improve the performance of the kit on ELISA automatic systems, it is recommended to increase the number of washes.
- The incomplete or inaccurate liquid removal from the wells could influence the assay precision and/or increase the background.
- It is important that the time of reaction in each well is held constant for reproducible results. Pipetting of samples should not extend beyond ten minutes to avoid assay drift. If more than 10 minutes are needed, follow the same order of dispensation. If more than one plate is used, it is recommended to repeat the dose response curve in each plate
- Addition of the TMB Substrate solution initiates a kinetic reaction, which is terminated by the addition of the Stop Solution. Therefore, the TMB Substrate and the Stop Solution should be added in the same sequence to eliminate any time deviation during the reaction.
- Observe the guidelines for performing quality control in medical laboratories by assaying controls and/or pooled sera.
- Maximum precision is required for reconstitution and dispensation of the reagents.
- Samples microbiologically contaminated, highly lipemeic or haemolysed should not be used in the assay.
- Plate readers measure vertically. Do not touch the bottom of the wells.

6. PROCEDURE

6.1. Preparation of the Calibrators (C_0…C_5)
Before use, mix for 5 minutes with a rotating mixer.
The Calibrators are ready for use and have the following concentration of Testosterone:

	C_0	C_1	C_2	C_3	C_4	C_5
pg/mL	0	0.2	1.0	4.0	20.0	100.0

Once opened, the Calibrators are stable 6 months at 2-8°C.

6.2. Preparation of the Sample
The determination of Free Testosterone can be performed in human serum or plasma.
Store specimen at -20°C if the determination is not performed on the same day of the sample collection. Avoid repetitive freezing and thawing of samples.

The Control is ready for use.

6.3. Preparation of the Wash Solution

Dilute the content of the vial "10X Conc. Wash Solution" with distilled water to a final volume of 500 mL prior to use. For smaller volumes respect the 1:10 dilution ratio. The diluted wash solution is stable for 30 days at 2÷8°C. In concentrated wash solution is possible to observe the presence of crystals, in this case mix at room temperature until complete dissolution of crystals; for greater accuracy dilute the whole bottle of concentrated wash solution to 500 mL on taking care also transfer the crystals, then mix until crystals are completely dissolved.

6.4. Procedure

- **Allow all reagents to reach room temperature (22-28°C) for at least 30 minutes.** At the end of the assay, store immediately the reagents at 2-8°C: avoid long exposure to room temperature.
- Unused coated microwell strips should be released securely in the foil pouch containing desiccant and stored at 2-8°C.
- To avoid potential microbial and/or chemical contamination, unused reagents should never be transferred into the original vials.
- As it is necessary to perform the determination in duplicate in order to improve accuracy of the test results, prepare two wells for each point of the calibration curve (C_0-C_5), two for each Control, two for each sample, one for Blank.

Reagent	Calibrator	Sample/ Control	Blank
Calibrator C_0-C_5	**20 µL**		
Sample/ Control		**20 µL**	
Conjugate	**100 µL**	**100 µL**	
Incubate at 37°C for 1 hour. Remove the content from each well; wash the wells 3 times with 300 µL of diluted wash solution (if you use automated equipment, wash the wells at least 5 times). **Important note**: during each washing step, gently shake the plate for 5 seconds and remove excess solution by tapping the inverted plate on an absorbent paper towel.			
TMB Substrate	**100 µL**	**100 µL**	**100 µL**
Incubate at room temperature (22÷28°C) for 15 minutes in the dark.			
Stop Solution	**100 µL**	**100 µL**	**100 µL**
Shake the meicroplate gently. Read the absorbance (E) at 450 nm against a reference wavelength of 620-630 nm or against Blank within 5 minutes.			

7. QUALITY CONTROL

Each laboratory should assay controls at normal, high and low levels range of Free Testosterone for monitoring assay performance. These controls should be treated as unknowns and values determined in every test procedure performed. Quality control charts should be maintained to follow the performance of the supplied reagents. Pertinent statistical methods should be employed to ascertain trends. The individual laboratory should set acceptable assay performance limits. Other parameters that should be monitored include the 80, 50 and 20% intercepts of the calibration curve for run-to-run reproducibility. In addition, maximum absorbance should be consistent with past experience. Significant deviation from established performance can indicate unnoticed change in experimental conditions or degradation of kit reagents. Fresh reagents should be used to determine the reason for the variations.

8. RESULTS

8.1. Mean Absorbance

Calculate the mean of the absorbance (Em) for each point of the calibration curve (C_0-C_5) and of each sample.

8.2. Calibration curve

Plot the mean value of absorbance (Em) of the Calibrators (C_0-C_5) against concentration. Draw the best-fit curve through the plotted points. (es: Four Parameter Logistic).

8.3. Calculation of Results

Interpolate the values of the samples on the calibration curve to obtain the corresponding values of the concentrations expressed in pg/mL.

9. REFERENCE VALUES

Serum concentrations of Free Testosterone are within the following ranges:

	Median	Mean ± 1SD pg/mL	Range pg/mL
Male	14	13,0 ± 7,0	4,5 - 42
Female			
Ovulating	1,3	1,4 ± 0,9	ND - 4,1
Oral contrceptives	0,9	1,1 ± 0,6	0,3 - 2,0
Post-menopausal	0,8	0,9 ± 0,5	0,1 - 1,7

Please pay attention to the fact that the determination of a range of expected values for a "normal" population in a given method is dependent on many factors, such as specificity and sensitivity of the method used and type of population under investigation. Therefore each laboratory should

consider the range given by the Manufacurer as a general indication and produce their own range of expected values based on the indigenous population where the laboratory works.

10. PERFORMANCE AND CHARACTERISTICS

10.1. Precision

10.1.1. Intra Assay Variation

Within run variation was determined by replicate (15x) the measurement of three different serum samples in the same assay. The within assay variability is ≤ 10%.

10.1.2. Inter Assay Variation

Between run variation was determined by replicate the measurement of five different control sera and two serum samples in 10 different assays. The between assay variability is ≤ 10%.

10.2. Sensitivity

The lowest detectable concentration of Free Testosterone that can be distinguished from the Calibrator 0 is 0.06 pg/mL.

10.3. Specificity

The specificity was assessed by measuring the apparent response of the assay to the following potentially cross-reactive analytes and interfering substances (anticoagulants).
The cross reaction of the antibody calculated at 50% according to Abraham are shown in the table:

Analyte	% Cross reactivity
Testosterone	100
DHT	0,00008
Androstenedione	0,0043
Androsterone	0,00029
DHEA-S	0,00007
Cortisol	< 0,00001
Cortisone	< 0,00001
17β Estradiol	0,00005
Estrone	< 0,00001
Prednisone	< 0,00001
17α Ethynilestradiol	< 0,00001
Norgestrel	0,00001
Danazol	< 0,00001
Aldosterone	< 0,00001
Sodium Citrate	< 0,00001
EDTA	< 0,00001
Heparin	< 0,00001

10.4. Correlation with RIA

Diametra Free Testosterone ELISA was compared to a commercially available Free Testosterone RIA (DPC-Coat a Count) kit. Serum samples of 24 females and 17 males were analysed according to both test systems.
The linear regression curve was calculated
(FT Diametra) = 0.957*(FT RIA) + 0.953
r^2 = 0.937

11. WASTE MANAGEMENT

Reagents must be disposed off in accordance with local regulations.

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ERROR POSSIBLE CAUSES / SUGGESTIONS

No colorimetric reaction

- no conjugate pipetted reaction after addition
- contamination of conjugates and/or of substrate
- errors in performing the assay procedure (e.g. accidental pipetting of reagents in a wrong sequence or from the wrong vial, etc.)

Too low reaction (too low ODs)

- incorrect conjugate (e.g. not from original kit)
- incubation time too short, incubation temperature too low

Too high reaction (too high ODs)

- incorrect conjugate (e.g. not from original kit)
- incubation time too long, incubation temperature too high
- water quality for wash buffer insufficient (low grade of deionization)
- insufficient washing (conjugates not properly removed)

Unexplainable outliers

- contamination of pipettes, tips or containers

insufficient washing (conjugates not properly removed) too high within-run

- reagents and/or strips not pre-warmed to CV% Room Temperature prior to use
- plate washer is not washing correctly (suggestion: clean washer head)

too high between-run - incubation conditions not constant (time, CV % temperature)

- controls and samples not dispensed at the same time (with the same intervals) (check pipetting order)
- person-related variation