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Please use only the valid version of the Instructions for Use provided with the kit

## Instructions for use

# Free Testosterone ELISA 2nd Generation

REF **AA E-1800R**

2°C – 8°C

Σ 96

RUO For research use only – Not for use in diagnostic procedures

**Free Testosterone ELISA** 2nd Generation

***INTENDED USE***
For the direct quantitative determination of Free Testosterone by an enzyme immunoassay in human serum.

***PRINCIPLE OF THE TEST***
The principle of the following enzyme immunoassay test follows the typical competitive binding scenario. Competition occurs between an unlabelled antigen (present in standards, controls and samples) and an enzyme-labelled antigen (conjugate) for a limited number of antibody binding sites on the microplate. 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 samples and controls can be directly read.
The *Free Testosterone ELISA* 2nd Generation kit 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.

***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; in women however, 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 Standard 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 wells 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.

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***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 60 pg/ml should not be diluted. Dilution will alter the equilibrium between free testosterone and serum proteins.

***SAFETY CAUTIONS AND WARNINGS***
***POTENTIAL BIOHAZARDOUS MATERIAL***

Human serum that may be used in the preparation of the standards and control 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. No test method however can offer complete assurance that HIV, HCV and Hepatitis B virus or any infectious agents are absent. The reagents should be considered as 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***

1. Precision pipettes to dispense 25, 50, 100, 150 and 350 µl
2. Disposable pipette tips
3. Distilled or deionized water
4. A 37 °C incubator
5. Microplate reader with a filter set at 450nm and an upper OD limit of 3.0 or greater* (see assay procedure step 11).

***REAGENTS PROVIDED***

**1. AA E-0030** WASH-CONC 10x **Wash Buffer Concentrate –** Requires Preparation **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. |

**2. 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. |

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

Contents: One vial containing 1 M sulfuric acid.

Volume: 6 ml/vial

Storage: Refrigerate at 2 – 8 °C

Stability: 12 months or as indicated on label.

Hazards identification:

H315 Causes skin irritation.
H319 Causes serious eye irritation.

**4. Standards** and **Controls –** Ready To Use

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

| Cat. no. | Symbol | Standard | Concentration* | Volume/Vial |
|---|---|---|---|---|
| **AA E-1801** | STANDARD A | Standard A | 0 pg/ml | 0.5 ml |
| **AA E-1802** | STANDARD B | Standard B | 0.1 pg/ml | 0.5 ml |
| **AA E-1803** | STANDARD C | Standard C | 1 pg/ml | 0.5 ml |
| **AA E-1804** | STANDARD D | Standard D | 5 pg/ml | 0.5 ml |
| **AA E-1805** | STANDARD E | Standard E | 20 pg/ml | 0.5 ml |
| **AA E-1806** | STANDARD F | Standard F | 60 pg/ml | 0.5 ml |
| **AA E-1851** | CONTROL 1 | Control 1 | Refer to vial labels for expected value and acceptable range! | 0.5 ml |
| **AA E-1852** | CONTROL 2 | Control 2 | | 0.5 ml |

Contents: 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, 0.1, 1, 5, 20 and 60 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.

**5. 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.

**6. AA E-1431** 96 **Rabbit Anti-Free Testosterone Antibody-Coated Break-Apart Well Microplate –** Ready To Use

Contents: One 96 well (12x8) polyclonal antibody-coated microplate in a resealable pouch with desiccant.

Storage: Refrigerate at 2 – 8 °C

Stability: 12 months or as indicated on label.

**7. AA E-1840** CONJUGATE-CONC 50x **Free Testosterone-Horseradish Peroxidase (HRP) Conjugate Concentrate –** Requires Preparation **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.

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***ASSAY PROCEDURE***

| Specimen Pretreatment: ***None.*** |
|---|

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 well strips. Reseal the bag and return any unused strips to the refrigerator. |
| **3.** | **Pipette 25 µl** of each **standard, control and specimen sample** into correspondingly labelled wells in duplicate. |
| **4.** | Pipette **100 µl** of the **conjugate working solution** into each well.<br>*(We recommend using a multichannel pipette).* |
| **5.** | Gently **shake** the plate for **10 seconds**. |
| **6.** | **Incubate the plate at 37 ºC** for **1 hour.** |
| **7.** | Wash the wells **<u>3 times</u> with 350 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 ºC** for **10 – 15 minutes**.<br>*(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 microplate 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 specimen/control samples.* |

***CALCULATIONS***

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 or 5-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***

Sample data only. **Do not** use to calculate results.

| Standard | Mean OD (450 nm) | Value (pg/ml) |
|---|---|---|
| A | 2.292 | 0 |
| B | 1.680 | 0.1 |
| C | 1.181 | 1 |
| D | 0.780 | 5 |
| E | 0.426 | 20 |
| F | 0.214 | 60 |
| Unknown | 1.066 | 1.59 |
| Unknown | 0.441 | 19.6 |

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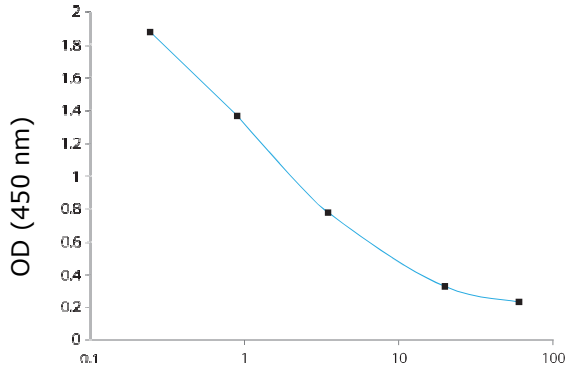
***TYPICAL STANDARD CURVE***
Sample curve only. **Do not** use to calculate results:




***PERFORMANCE CHARACTERISTICS***
***SENSITIVITY***
The limit of detection (LoD) was determined from the analysis of 64 replicates of a low value sample and from the LoB.
LoD = LoB + 1.645σS,
where σS is the standard deviation of the low value sample. σS was determined to be 0.0093 based on 64 measurements of a low value sample.

LoD = 0.0025 + (1.645*0.0093) = **0.018 pg/ml**.

***SPECIFICITY (CROSS REACTIVITY)***
The following compounds were tested for cross-reactivity with the **Free Testosterone ELISA** **2nd Generation** kit with testosterone cross-reacting at 100%.

| Steroid | % Cross Reactivity |
|---|---|
| Testosterone | 100 |
| 5α-DHT | 3.5 |
| Androstenedione | 0.17 |
| Progesterone | 0.007 |
| Androsterone | 0.075 |
| Aldosterone | <0.008 |
| Cholesterol | <0.0001 |
| Cortisone | 0.0025 |
| DHEA | 0.071 |
| DHEAS | 0.0014 |
| 17β-Estradiol | 0.15 |
| Estriol | <0.008 |
| Pregnenolone | 0.028 |

***INTRA-ASSAY PRECISION***
Five samples were assayed 24 times each on the same standard curve. The results (in pg/ml) are tabulated below:

| Sample | Mean | CV % |
|---|---|---|
| 1 | 2.24 | 6.7 |
| 2 | 3.81 | 6.4 |
| 3 | 13.6 | 6.0 |
| 4 | 13.7 | 5.9 |
| 5 | 23.7 | 4.8 |

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***INTER-ASSAY PRECISION***

Three samples were assayed twenty times in duplicate over a period of greater than ten days.
The results (in pg/ml) are tabulated below:

| Sample | Mean | CV % |
|---|---|---|
| 1 | 3.53 | 8.1 |
| 2 | 13.8 | 11.5 |
| 3 | 23.3 | 6.9 |

***COMPARATIVE STUDIES***

The *Free Testosterone ELISA* *2nd Generation* Kit (y) was compared with a competitors Free Testosterone Coated Tube RIA Kit (x). The comparison of 60 serum samples yielded the following linear regression results:

y = 0.9362x (competitor) + 3.8794, r = 0.97

***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-HRP conjugate. A charcoal-stripped human serum pool was spiked precisely with SHBG at concentrations ranging from 6.25 **–** 200 µg/ml and was assayed with the *Free Testosterone ELISA* 2nd Generation Kit. Results tabulated below (in pg/ml):

| SHBG Added | OD 450nm | Percent B/$B_o$ (%) |
|---|---|---|
| 0 | 2.37 | 100.0 |
| 6.25 | 2.37 | 99.9 |
| 12.5 | 2.34 | 98.7 |
| 50 | 2.36 | 99.5 |
| 200 | 2.27 | 95.6 |

The results showed % binding values between 95 **–** 100% (Bo = unspiked serum) even at higher than normal SHBG levels. In conclusion, the results showed that there was no significant binding by SHBG on the free testosterone-HRP conjugate.

***EXPECTED NORMAL VALUES***

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):

| Cohort Group; Gender/Age | N | 95% Confidence Range | Absolute Range |
|---|---|---|---|
| Males / < 13 | 44 | – | ND – 1.6 |
| Males / 13 – 19 | 37 | – | ND – 22.3 |
| Males /20 – 39 | 120 | 9.1 – 32.2 | – |
| Males / 40 – 59 | 120 | 5.7 – 30.7 | – |
| Males / ≥ 60 | 120 | 5.9 – 27.0 | – |
| Females / < 13 | 63 | – | ND – 1.3 |
| Females / 13 – 19 | 17 | – | 0.2 – 2.0 |
| Females / 20 – 39 | 120 | 0.1 – 6.3 | – |
| Females / 40 – 59 | 120 | 0.2 – 4.1 | – |
| Females / ≥ 60 | 60 | 0.5 – 3.9 | – |

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***CHANGE HISTORY***

| | | | |
|---|---|---|---|
| **Previous Version:** | 8.0-r | **New Version:** | 8.0a-r |
| **Changes:** | ***REAGENTS PROVIDED***<br>Hazard labelling for component AA E-0080 updated | | |

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**Symbols:**

| | | | | | |
|---|---|---|---|---|---|
| | Storage temperature | | Manufacturer | | Contains sufficient for <n> tests |
| | Use-by date | LOT | Batch code | | |
| | Consult instructions for use | CONT | Content | | |
| | Caution | REF | Catalogue number | | Distributor |
| | Date of manufacture | | | RUO | For research use only! |