Testosterone
Enzyme Immunoassay
Test Kit

Intended Use
For the quantitative determination of testosterone concentration in human serum. For in vitro diagnostic use only.

Introduction
Testosterone (17β-hydroxyandrost-4-ene-3-one) is a C19 steroid with an unsaturated bond between C-4 and C-5, a ketone group in C-3 and a hydroxyl group in the β position at C-17. This steroid hormone has a molecular weight of 288.4. Testosterone is the most important androgen secreted into the blood. In males, testosterone is secreted primarily by the Leydig cells of the testes; in females, 50% of circulating testosterone is derived from peripheral conversion of androstenedione, 25% from the ovary and 25% from the adrenal glands.

Testosterone is responsible for the development of secondary male sex characteristics and its measurements are helpful in evaluating the hypogonadal states.

In women, high levels of testosterone are generally found in hirsutism and virilization, polycystic ovaries, ovarian tumors, adrenal tumors and adrenal hyperplasia.

In men, high levels of testosterone are associated with testicular tumors, congenital adrenal hyperplasia and prostate cancer.

Low levels of testosterone can be found in patients with the following diseases: Hypopituitarism, Klinefelter's syndrome, Testicular feminization, Orchidectomy and Cryptorchidism, enzymatic defects and some autoimmune diseases.

The testosterone EIA kit is designed for the measurement of total testosterone in human serum.

Principle of the Test
The testosterone EIA is based on the principle of competitive binding between testosterone in the test specimen and testosterone-HRP conjugate for a constant amount of rabbit anti-testosterone. In the incubation, goat anti-rabbit IgG-coated wells are incubated with 10µl of testosterone standards, controls, patient samples, 100 µl testosterone-HRP conjugate reagent and 50µl rabbit anti-testosterone reagent at 37°C for 90 minutes. During the incubation, a fixed amount of HRP-labeled testosterone competes with the endogenous testosterone in the standard, sample, or quality control serum for a fixed number of binding sites of the specific testosterone antibody. Thus, the amount of testosterone peroxidase conjugate immunologically bound to the well progressively decreases as the concentration of Testosterone in the specimen increases. Unbound testosterone peroxidase conjugate is then removed and the wells washed. Next, a solution of TMB reagent is then added and incubated at room temperature for 20 minutes, resulting in the development of blue color. The color development is stopped with the addition of 1N HCl, and the absorbance is measured spectrophotometrically at 450nm.

Storage of Test Kit and Instrumentation
Unopened test kits should be stored at 2-8°C for up to 24 hours, and should be frozen at −10°C or lower for longer periods. Do not use grossly hemolyzed or grossly lipemic specimens.

Reagents

Materials provided with the kit:
1. Goat Anti-Rabbit IgG-coated microtiter wells, 96 wells
2. Testosterone reference standards: 0, 0.1, 0.5, 2.0, 6.0 and 18.0ng/ml.
   Liquids 0.5ml each, ready to use.
3. Rabbit anti-testosterone reagent (pink color), 7 ml
4. Testosterone-HRP conjugate reagent (blue color), 12 ml
5. Testosterone control 1. Liquid, 0.5 ml, ready to use
6. Testosterone control 2. Liquid, 0.5 ml, ready to use
7. TMB reagent (One-Step) 11 ml
8. Stop solution (1N HCl), 11 ml

Materials required but not provided:
1. Precision pipettes: 10 µl, 50 µl, 100 µl, and 1.0 ml
2. Disposable pipette tips
3. Distilled or deionized water
4. Vortex mixer or equivalent
5. Absorbent paper or paper towel
6. Linear-linear graph paper
7. Microtiter plate reader

Warnings and Precautions for Users
Test methods are not available which can offer complete assurance that Hepatitis B virus, Human Immunodeficiency Virus (HIV/HTLV-III/LAV), or other infectious agents are absent from the reagents in this kit. Therefore, all human blood products, including patient samples, should be considered potentially infectious. Handling and disposal should be in accordance with the procedures defined by an appropriate national biohazard safety guideline or regulation, where it exists (e.g., USA Center for Disease Control/National Institute of Health Manual, "Biosafety in Microbiological and Biomedical Laboratories," 1984).

Specimen Collection and Preparation
No special pretreatment of sample is necessary. Serum samples may be stored at 2-8°C for up to 24 hours, and should be frozen at −10°C or lower for longer periods. Do not use grossly hemolyzed or grossly lipemic specimens.

Note: Samples containing sodium azide should not be used in the assay.

Assay Procedure
1. Secure the desired number of coated wells in the holder.
2. Dispense 10 µl of standards, specimens, and controls into appropriate wells.
3. Dispense 100 µl of Testosterone-HRP conjugate reagent into each well.
4. Dispense 50 µl of rabbit anti-testosterone reagent to each well.
5. Incubate at 37°C for 90 minutes. Thoroughly mix for 30 seconds. It is very important to mix completely.

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6. Rinse and flick the microwells 5 times with distilled or deionized water. (Do not use tap water).
7. Dispense 100 µl of TMB Reagent into each well. Gently mix for 10 seconds.
8. Incubate at room temperature (18-25°C) for 20 minutes.
9. Stop the reaction by adding 100µl of Stop Solution to each well.
10. Gently mix 30 seconds. It is important to make sure that all the blue color changes to yellow color completely.
11. Read absorbance at 450 nm with a microtiter well reader within 15 minutes.

Calculation of Results
1. Calculate the mean absorbance value (A450) for each set of reference standards, controls and samples.
2. Construct a standard curve by plotting the mean absorbance obtained for each reference standard against its concentration in ng/ml on a linear-linear graph paper, with absorbance values on the vertical or Y axis, and concentrations on the horizontal or X axis.
3. Use the mean absorbance values for each specimen to determine the corresponding concentration of testosterone in ng/ml from the standard curve.
4. Any values obtained for diluted samples must be further converted by applying the appropriate dilution factor in the calculations.

Example of Standard Curve
Results of a typical standard run with optical density readings at 450nm shown in the Y axis against testosterone concentrations shown in the X axis.

Note: This standard curve is for the purpose of illustration only, and should not be used to calculate unknowns. Each laboratory must provide its own data and standard curve in each experiment.

Expected Normal Values
Each laboratory should establish its own normal range based on the patient population. The testosterone EIA was performed on randomly selected outpatient clinical laboratory samples. The results of these determinations are as follows:

Males:  
- prepubertal (late) 0.1 – 0.2 ng/ml
- Adult 3.0 – 10.0 ng/ml

Females:  
- prepubertal (late) 0.1 – 0.2 ng/ml
- follicular phase 0.2 – 0.8 ng/ml
- luteal phase 0.2 – 0.8 ng/ml
- post menopausal 0.08 – 0.35 ng/ml

Sensitivity
The minimum detectable concentration of the Testosterone ELISA assay as measured by 2SD from the mean of a zero standard is estimated to be 0.05 ng/ml.

Precision

Intra-Assay Precision
Within run precision was determined by replicate determinations of four different serum samples in one assay. Within assay variability is shown below:

<table>
<thead>
<tr>
<th>Samples</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td># Replicates</td>
<td>24</td>
<td>24</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>Mean Testosterone (ng/ml)</td>
<td>0.44</td>
<td>3.7</td>
<td>5.1</td>
<td>12.7</td>
</tr>
<tr>
<td>S.D.</td>
<td>0.03</td>
<td>0.4</td>
<td>0.4</td>
<td>0.6</td>
</tr>
<tr>
<td>C.V. %</td>
<td>6.4</td>
<td>10.0</td>
<td>8.3</td>
<td>5.0</td>
</tr>
</tbody>
</table>

Inter-Assay Precision
Between run precision was determined by replicate measurements of six different serum samples over a series of individually calibrated assays. Between assay variability is shown below:

<table>
<thead>
<tr>
<th>Samples</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td># Replicates</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Mean Testosterone (ng/ml)</td>
<td>0.45</td>
<td>3.4</td>
<td>5.0</td>
<td>13.3</td>
</tr>
<tr>
<td>S.D.</td>
<td>0.02</td>
<td>0.3</td>
<td>0.2</td>
<td>0.5</td>
</tr>
<tr>
<td>C.V. %</td>
<td>4.4</td>
<td>8.4</td>
<td>4.4</td>
<td>3.7</td>
</tr>
</tbody>
</table>

Recovery Study
Various patient serum samples of known Testosterone levels were combined and assayed in duplicate. The mean recovery was 95.3%.

<table>
<thead>
<tr>
<th>Pair No.</th>
<th>Expected Testosterone (ng/ml)</th>
<th>Observed Testosterone (ng/ml)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.7</td>
<td>9.2</td>
<td>105.9</td>
</tr>
<tr>
<td>2</td>
<td>9.3</td>
<td>9.6</td>
<td>103.6</td>
</tr>
<tr>
<td>3</td>
<td>6.3</td>
<td>5.2</td>
<td>83.2</td>
</tr>
<tr>
<td>4</td>
<td>5.0</td>
<td>5.0</td>
<td>99.9</td>
</tr>
<tr>
<td>5</td>
<td>2.6</td>
<td>3.3</td>
<td>127.5</td>
</tr>
<tr>
<td>6</td>
<td>2.4</td>
<td>2.3</td>
<td>97.5</td>
</tr>
<tr>
<td>7</td>
<td>0.66</td>
<td>0.46</td>
<td>70.4</td>
</tr>
<tr>
<td>8</td>
<td>0.61</td>
<td>0.46</td>
<td>74.6</td>
</tr>
</tbody>
</table>
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Specificity

The following materials have been checked for cross reactivity. The percentage indicates cross reactivity at 50% displacement compared to Testosterone.

Data on the cross-reactivity for several endogenous and pharmaceutical steroids are summarized in the following table:

Cross-reactivity (%) = \( \frac{\text{Observed Testosterone Concentration}}{\text{Steroid Concentration}} \times 100 \)

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Cross-Reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone</td>
<td>100%</td>
</tr>
<tr>
<td>Dihydrotestosterone</td>
<td>0.86%</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>0.89%</td>
</tr>
<tr>
<td>Androsterone</td>
<td>1.0%</td>
</tr>
<tr>
<td>17(\beta) Estradiol</td>
<td>0.05%</td>
</tr>
<tr>
<td>Progesterone</td>
<td>&lt;0.05%</td>
</tr>
<tr>
<td>Epitestosterone</td>
<td>&lt;0.05%</td>
</tr>
<tr>
<td>17-OH-Progesterone</td>
<td>&lt;0.05%</td>
</tr>
<tr>
<td>Estriol</td>
<td>&lt;0.05%</td>
</tr>
<tr>
<td>Cortisol</td>
<td>&lt;0.05%</td>
</tr>
<tr>
<td>DHEA-Sulphate</td>
<td>&lt;0.05%</td>
</tr>
</tbody>
</table>

Clinical Application

Information is cited from reference #7

In Males:

In man, the determination of testosterone is used as an indicator for the function of the testes: low hormone levels are found in cases with Klinefelter’s syndrome, cryptorchism or anorchia. Males with testosterone deficiency often present with a number of symptoms such as decreased libido, as well as decreased muscle strength, gynecomastia and infertility.

In Females:

Virilizing Disorders:

Testosterone measurements are frequently utilized in the evaluation of virilizing disorders. **Testosterone concentrations >2.0 ng/ml may indicate androgen secreting ovarian or adrenal neoplasms.**

Monitoring of Androgen Suppressing Drugs:

Testosterone measurements may be utilized in women for the adjustment of androgen suppressing drugs and their dosages.

Pregnancy:

Testosterone concentrations are relatively consistent during pregnancy.

Limitations of the Procedure

1. Reliable and reproducible results will be obtained when the assay procedure is carried out with a complete understanding of the package insert instructions and with adherence to good laboratory practice.
2. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.

3. Serum samples demonstrating gross lipemia, gross hemolysis, or turbidity should not be used with this test.
4. The results obtained from the use of this kit should be used only as an adjunct to other diagnostic procedures and information available to the physician.

Quality Control

Good laboratory practice requires that controls are run with each calibration curve. A statistically significant number of controls should be assayed to establish mean values and acceptable ranges to assure proper performance. The testosterone EIA kit includes internal controls, Level 1 and 2.

References


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