INTENDED USE

For the direct quantitative determination of 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 patient 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 amount of the antigen in the sample. A set of standards is used to plot a standard curve from which the amount of testosterone in patient samples and controls can be directly read.

CLINICAL APPLICATIONS

Testosterone is the most important male sex hormone, it is responsible for normal steroid biosynthesis. In females small amounts of testosterone are produced by the ovary. Serum or plasma levels is an index of leydig cell function and controls can be directly read.

6. A calibration 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 arbitrary 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 in 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 lots of kits components within a test and do not use any component beyond the expiration date on the label.
14. Kit reagents must be regarded as hazardous waste and disposed of according to national regulations.

LIMITATIONS

1. All the reagents within the kit are calibrated for the direct determination of testosterone in human serum. The kit is not calibrated for the determination of testosterone in saliva, plasma or 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. Only calibrator A may be used to dilute any high serum sample. The use of any other reagent may lead to false results.
5. 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.

SAFETY 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 before use. Avoid repeated freezing and thawing of reagents and specimens.

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 0.2 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 50, 100, 150 and 300 µL
2. Disposable pipette tips
3. Distilled or deionized water
4. Plate shaker
5. Microplate reader with a filter set at 450 nm and an upper OD limit of 3.0 or greater

REAGENTS PROVIDED

1. Rabbit Anti-Testosterone Antibody-Coated Break-Apart Well Microplate — Ready To Use
   Contents: One 96-well (128) polyclonal antibody-coated microplate in a resealable pouch with desiccant.
   Storage: Refrigerate at 2–8°C
   Stability: 12 months or as indicated on label.

2. Testosterone-Horseradish Peroxidase (HRP) Conjugate Concentrate — Requires Preparation
   Contents: Testosterone-HRP conjugate in a protein-based buffer with a non-mercury preservative.
   Volume: 0.5 mL/vial
   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. The whole plate is to be used diluted 1:100, store diluted buffer concentrate in 450 mL of water.

3. Testosterone Calibrators — Ready To Use
   Contents: Six vials containing testosterone in a human serum-based buffer with a non-mercury preservative.
   Volume: 0.5 mL of assay buffer
   Stability: 12 months or as indicated on label.

4. Calibrator A
   Concentration: 0 ng/mL
   Volume/mL: 1.0 mL

5. Calibrator B
   Concentration: 0.08 ng/mL
   Volume/mL: 0.5 mL

6. Calibrator C
   Concentration: 0.42 ng/mL
   Volume/mL: 0.5 mL

7. Calibrator D
   Concentration: 1.67 ng/mL
   Volume/mL: 0.5 mL

8. Calibrator E
   Concentration: 5.0 ng/mL
   Volume/mL: 0.5 mL

9. Calibrator F
   Concentration: 16.7 ng/mL
   Volume/mL: 0.5 mL

Stability: 12 months in unopened vials or as indicated on label. Once opened, the controls should be used within 14 days or aliquoted and stored frozen. Avoid multiple freezing and thawing cycles.

5. Wash Buffer Concentrate — Requires Preparation
   Contents: One bottle containing buffer with a non-ionic detergent and a non-mercury preservative.
   Volume: 50 mL
   Storage: Refrigerate at 2–8°C
   Stability: 12 months or as indicated on label.

6. Assay Buffer — Ready To Use
   Contents: One bottle containing a protein-based buffer with a non-mercury preservative.
   Volume: 15 mL/bottle
   Storage: Refrigerate at 2–8°C
   Stability: 12 months or as indicated on label.

7. TMB Substrate — Ready To Use
   Contents: One bottle containing tetramethylbenzidine and hydrogen peroxide in a non-OMF or DMSO containing buffer.
   Volume: 16 mL/bottle
   Storage: Refrigerate at 2–8°C
   Stability: 12 months or as indicated on label.

8. Stopping Solution — Ready To Use
   Contents: One bottle containing 1M sulfuric acid.
   Volume: 6 mL/bottle
   Storage: Refrigerate at 2–8°C
   Stability: 12 months or as indicated on label.
1. Prepare working solutions of the testosterone-HRP conjugate and wash buffer.
2. Remove the required number of well strips. Reserve the bag and return any unused strips to the refrigerator.
3. Pipette 50 μL of each calibrator, control and specimen sample into corresponding labelled wells in duplicate. The optical densities will be lower, however, if the 450 nm filter is unavailable, a 405 or 415 nm filter may be recommended.
4. Incubate on a plate shaker for 10–15 minutes at room temperature (or until calibrator A attains dark blue colour for desired OD).
5. Pipette 150 μL of TMB substrate into each well at timed intervals as in step 7.
6. Wash the wells 3 times with 300 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.)
7. Incubate on a plate shaker (approximately 200 rpm) for 1 hour at room temperature.
8. Wash the wells 3 times with 300 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.)
9. Pipette 50 μL of stopping solution into each well at the same timed intervals as in step 7.
10. Read the plate on a microplate reader at 450 nm within 20 minutes after addition of the stopping solution.

**CALCULATIONS**

1. Calculate the mean optical density of each calibrator duplicate.
2. Draw a calibration curve on semi-log paper with the X-axis as the concentrations on the CALIBRATORS and the Y-axis as the mean optical density (OD). 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 calibration curve.
5. If the sample reads more than 20 ng/mL, then dilute it with calibrator A at a dilution of no more than 1:8. The result obtained should be multiplied by the dilution factor.

**ASSAY PROCEDURE**

**Specimen Pretreatment:** None.

All reagents must reach room temperature before use. Calibrators, controls and specimen samples should be assayed in duplicate. Once the procedure has been started, all steps should be completed without interruption.

- **Calibrator OD:** 2.391
- **OD 2:** 2.357
- **Mean OD:** 2.374
- **Value (ng/mL):** 2
- **Calibrator:** 1.253
- **OD:** 1.576
- **Mean OD:** 1.566
- **Value (ng/mL):** 0.5
- **Calibrator:** 0.984
- **OD:** 1.039
- **Mean OD:** 1.012
- **Value (ng/mL):** 2
- **Calibrator:** 0.686
- **OD:** 0.575
- **Mean OD:** 0.591
- **Value (ng/mL):** 6
- **Calibrator:** 0.200
- **OD:** 0.283
- **Mean OD:** 0.292
- **Value (ng/mL):** 2
- **Calibrator:** 1.286
- **OD:** 1.238
- **Mean OD:** 1.252
- **Value (ng/mL):** 1

**TYPICAL TABULATED DATA**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean</th>
<th>SD</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.76</td>
<td>0.07</td>
<td>9.6</td>
</tr>
<tr>
<td>2</td>
<td>0.77</td>
<td>0.06</td>
<td>7.7</td>
</tr>
<tr>
<td>3</td>
<td>1.37</td>
<td>0.08</td>
<td>6.6</td>
</tr>
</tbody>
</table>

**INTER-ASSAY PRECISION**

Three samples were assayed ten times over a period of four weeks. The results (in ng/mL) are tabulated below:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean</th>
<th>SD</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.76</td>
<td>0.05</td>
<td>6.1</td>
</tr>
<tr>
<td>2</td>
<td>3.29</td>
<td>0.28</td>
<td>8.5</td>
</tr>
<tr>
<td>3</td>
<td>4.11</td>
<td>0.30</td>
<td>7.3</td>
</tr>
</tbody>
</table>

**RECOVERY**

Spiked samples were prepared by adding defined amounts of testosterone to four patient serum samples. The results (in ng/mL) are tabulated below:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Obs. Result</th>
<th>Exp. Result</th>
<th>Recover</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.40</td>
<td>1.37</td>
<td>101.2</td>
</tr>
<tr>
<td>2</td>
<td>7.13</td>
<td>8.07</td>
<td>84.3</td>
</tr>
<tr>
<td>3</td>
<td>4.67</td>
<td>4.82</td>
<td>97.0</td>
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</tbody>
</table>

**TYPICAL CALIBRATOR CURVE**

The calibrator curve on semi-log paper with the X-axis as the concentrations on the CALIBRATORS and the Y-axis as the mean optical densities on the Y-axis and the calibrator concentrations on the X-axis. If immunoassay software is being used, a 4-parameter or 5-parameter curve is recommended.

**PERFORMANCE CHARACTERISTICS**

**SENSITIVITY**

The lower detection limit is calculated from the standard curve by determining the resulting concentration of the mean OD of Calibrator A (based on 10 replicate analyses) minus 2 SD. Therefore, the sensitivity of the DBC Direct Testosterone ELISA kit is 0.022 ng/mL.

**SPECIFICITY (CROSS-REACTIVITY)**

The following compounds were tested for cross-reactivity with testosterone. As for all clinical assays each laboratory should collect data on sensitivity and specificity of immunoassays. Therefore, the sensitivity of the DBC Direct Testosterone ELISA kit is 0.022 ng/mL.

<table>
<thead>
<tr>
<th>Compound</th>
<th>% Cross Reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone</td>
<td>100.0</td>
</tr>
<tr>
<td>5α-DHT</td>
<td>1.4</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>0.8</td>
</tr>
<tr>
<td>Progesterone</td>
<td>0.5</td>
</tr>
<tr>
<td>Androsterone</td>
<td>0.1</td>
</tr>
</tbody>
</table>

**COMPARATIVE STUDIES**

The DBC Direct Testosterone ELISA kit (x) was compared with a competitors Testosterone ELISA kit (y). The comparison results: y = 1.4124x + 0.099, r = 0.96

**EXPECTED NORMAL VALUES**

As for all clinical assays each laboratory should collect data and publish their 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 ng/mL):

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean (ng/mL)</th>
<th>Central 95% (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prepubertal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infants</td>
<td>10</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.05–0.25</td>
</tr>
<tr>
<td>Puberty males</td>
<td>40</td>
<td>4.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.0–12.0</td>
</tr>
<tr>
<td>Females</td>
<td>40</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.2–1.0</td>
</tr>
</tbody>
</table>

**REFERENCES**


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SYMBOLS

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Meaning</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVD</td>
<td>In vitro diagnostic device</td>
</tr>
<tr>
<td>STG</td>
<td>Storage Temperature</td>
</tr>
<tr>
<td>Consult</td>
<td>Consult</td>
</tr>
<tr>
<td>Legal</td>
<td>Legal</td>
</tr>
<tr>
<td>Manufacturer</td>
<td>Manufacturer</td>
</tr>
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</table>

**LOT**

<table>
<thead>
<tr>
<th>Lot number</th>
<th>Expiry</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2/2</td>
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</tbody>
</table>