3. Users should have a thorough understanding of this protocol.

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**INTENDED USE**

For the direct quantitative determination of Androstenedione in human serum by an enzyme immunoassay.

**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.

**CLINICAL APPLICATIONS**

Androstenedione is produced by the adrenals and gonads. As a result, the determination of the level of androstenedione in serum is important in the evaluation of the functional state of the adrenals and gonads. Androstenedione is a precursor of testosterone. Studies on androstenedione have been shown to be an important source of androgens in human serum. Androstenedione is produced by the adrenals and gonads. As a result, the determination of the level of androstenedione in serum is important in the evaluation of the functional state of the adrenals and gonads. 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.

**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 hazardous 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 calibrator curve must be established for every run. The controls should be included in every run and fall within established confidence limits.

7. Improper procedural techniques, imprecise pipetting, incomplete washing as well as improper reactor storage may be indicated when assay values for the controls do not reflect established ranges.

8. 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.

9. To prevent contamination of reagents, use a new disposable pipette tip for dispensing each reagent, sample, standard and control.

10. 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.

11. 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 androstenedione in human serum. The kit is not calibrated for the determination of androstenedione 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 unsuitable for this test, as they may lead to false results.

4. Only calibrator A may be used to dilute any high serum standards. 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 heterophile antibodies in patients regularly exposed to antigenic protein preparations, etc., may influence the interpretation of results. Consequently, the clinical diagnosis should include all aspects of a patient’s medical history including the frequency of exposure to animals/plants/products if false results are suspected.

**SAFETY CAUTIONS AND WARNINGS**

**POTENTIAL BIOHAZARDOUS MATERIAL**

Human serum that may be used in the preparation of the standards and control has been tested for the following antigens: Hepatitis B surface antigen and has been found to be reactive for Hepatitis B surface antigen and Hepatitis B virus. The reagents should be considered a 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 any infectious agents are absent. The reagents have been shown to be an important source of androgens in human serum. Androstenedione is produced by the adrenals and gonads. As a result, the determination of the level of androstenedione in serum is important in the evaluation of the functional state of the adrenals and gonads. Androstenedione is produced by the adrenals and gonads. As a result, the determination of the level of androstenedione in serum is important in the evaluation of the functional state of the adrenals and gonads. 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.

**REAGENTS PROVIDED**

1. Rabbit Anti-Androstenedione Antibody-Coated BIA-Free 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.

2. Androstenedione-Horseradish Peroxidase (HRP) Conjugate — Ready To Use

- Contents: One bottle containing Androstenedione-HRP conjugate in a protein-based buffer with a non-mercury preservative.
- Storage: Refrigerate at 2–8°C.
- Volume: 14 mL/bottle
- Stability: 12 months or as indicated on label.

3. Androstenedione Calibrators — Ready To Use

- Contents: Six vials containing androstenedione in a human serum-based buffer with a non-mercury preservative. Prepared by spiking serum with a precise quantity of androstenedione.

**SPECIFIC COLLECTION AND STORAGE**

Approximately 0.1 mL of serum is required per duplicate determination. Collect 4–5 mL of blood into an appropriately labeled 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.

**PRETEST PRETREATMENT**

This assay is a direct system. No specimen pretreatment is necessary.

**EQUIPMENT NEEDED**

1. Precision pipettes to dispense 25, 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** (see assay procedure step 10).

**PROVIDED**

- Wash Buffer Concentrate — Ready To Use

- Contents: One bottle containing tetramethylbenzidine and hydrogen peroxide in a non-DMF or DMSO containing buffer.
- Storage: 2–8°C.
- Volume: 16 mL/bottle
- Stability: 12 months or as indicated on label.

- 5. Wash Buffer Concentrate — Requires Preparation

- Contents: One bottle containing a non-ionic detergent and a non-mercury preservative.
- Storage: 2–8°C.
- Volume: 50 mL/bottle
- Stability: 12 months or as indicated on label.

- Preparation: Dilute wash buffer concentrate 1:10 in distilled or deionized water before use. If the whole plate is to be washed, prepare a dilute 50x of wash buffer concentrate in 450 mL of water.

**LIMITATIONS**

1. 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.

2. Androstenedione-Horseradish Peroxidase (HRP)

- Conjugate — Ready To Use

- Contents: One bottle containing Androstenedione-HRP conjugate in a protein-based buffer with a non-mercury preservative.
- Storage: Refrigerate at 2–8°C.
- Volume: 14 mL/bottle
- Stability: 12 months or as indicated on label.

3. Androstenedione Calibrators — Ready To Use

- Contents: Six vials containing androstenedione in a human serum-based buffer with a non-mercury preservative. Prepared by spiking serum with a precise quantity of androstenedione.

**CALIBRATOR**

- Contents: Two vials containing androstenedione in a human serum-based buffer with a non-mercury preservative.

- Preparation: Spiking serum with a precise quantity of androstenedione. Refer to vial labels for expected value and acceptable range.

- Volume: 0.5 mL/vial

- Storage: Refrigerate at 2–8°C.

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

- 5. Wash Buffer Concentrate — Requires Preparation

- Contents: One bottle containing tetramethylbenzidine and hydrogen peroxide in a non-DMF or DMSO containing buffer.
- Storage: 2–8°C.
- Volume: 50 mL/bottle
- Stability: 12 months or as indicated on label.

- Preparation: Dilute wash buffer concentrate 1:10 in distilled or deionized water before use. If the whole plate is to be washed, prepare a dilute 50x of wash buffer concentrate in 450 mL of water.

**CONTROLS**

- Contents: Two vials containing androstenedione in a human serum-based buffer with a non-mercury preservative.

- Preparation: Spiking serum with a precise quantity of androstenedione. Refer to vial labels for expected value and acceptable range.

- Volume: 0.5 mL/vial

- Storage: Refrigerate at 2–8°C.

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

- 5. Wash Buffer Concentrate — Requires Preparation

-Contents: One bottle containing tetramethylbenzidine and hydrogen peroxide in a non-DMF or DMSO containing buffer.
- Storage: 2–8°C.
- Volume: 50 mL/bottle
- Stability: 12 months or as indicated on label.

- Preparation: Dilute wash buffer concentrate 1:10 in distilled or deionized water before use. If the whole plate is to be washed, prepare a dilute 50x of wash buffer concentrate in 450 mL of water.

- 6. TMB Substrate — Ready To Use

- Contents: One bottle containing 1% sulfuric acid in 95% ethanol.
- Storage: 2–8°C.
- Volume: 6 mL/bottle
- Stability: 12 months or as indicated on label.

- 7. Stopping Solution — Ready To Use

- Contents: Six vials containing androstenedione in a human serum-based buffer with a non-mercury preservative.

- Preparation: Spiking serum with a precise quantity of androstenedione.

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

1. Prepare working solutions of the wash buffer.
2. Remove the required number of wash strips. Reisele the bag and return any unused strips to the refrigerator.
3. Pipette 25 μL of each calibrator, control and specimen sample into correspondingly labelled wells in duplicate.
4. Pipette 100 μL of the Androstenedione-HPG conjugate into each well. (We recommend using a multichannel pipette.)
5. Incubate on a plate shaker (approximately 200 rpm) for 1 hour at room temperature.
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. Pipette 150 μL of TMB substrate into each well at the same timed intervals as in step 7.
8. Incubate on a plate shaker for 10–20 minutes at room temperature (or until calibrator A attains dark blue color for desired OD).
9. Wash the plate using 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 calibrator curve on semi-log paper with the mean optical densities on the Y-axis and the calibrator concentrations on the X-axis. If immunooassay software is being used, a 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 calibrator curve.
5. Calculate the mean optical density of each unknown duplicate.
6. Read the values of the unknowns directly off the calibrator curve.
7. Wash the wells 3 times with 300 μL of diluted wash buffer per well.
8. Incubate on a plate shaker (approximately 200 rpm) for 1 hour at room temperature.
9. Wash the plate using a microplate reader at 450 nm within 20 minutes after addition of the stopping solution.

SPECFICITY (CROSS-REACTIVITY)

The following compounds were tested for cross-reactivity with Androstenedione to three patient serum samples. The results (in ng/mL) are tabulated below:

<table>
<thead>
<tr>
<th>Compound</th>
<th>Value (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Androstenedione</td>
<td>100</td>
</tr>
<tr>
<td>DHEA</td>
<td>1.8</td>
</tr>
<tr>
<td>Testosterone</td>
<td>0.1</td>
</tr>
<tr>
<td>Estrone</td>
<td>0.1</td>
</tr>
<tr>
<td>Estradiol</td>
<td>0.1</td>
</tr>
<tr>
<td>Progesterone</td>
<td>0.1</td>
</tr>
<tr>
<td>17α-Hydroxyprogesterone</td>
<td>0.1</td>
</tr>
<tr>
<td>5α-DHT</td>
<td>0.1</td>
</tr>
<tr>
<td>Cortisol</td>
<td>0.01</td>
</tr>
<tr>
<td>DHEA-S</td>
<td>0.01</td>
</tr>
</tbody>
</table>

EXPECTED NORMAL VALUES

As for all clinical assays each laboratory should conduct data and establish their own range of expected normal values.

REFERENCE:


TYPICAL TABULATED DATA

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean OD Value (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2.443</td>
</tr>
<tr>
<td>B</td>
<td>1.766</td>
</tr>
<tr>
<td>C</td>
<td>1.195</td>
</tr>
<tr>
<td>D</td>
<td>0.721</td>
</tr>
<tr>
<td>E</td>
<td>0.385</td>
</tr>
<tr>
<td>F</td>
<td>0.184</td>
</tr>
<tr>
<td>G</td>
<td>0.482</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>A</td>
<td>0.528</td>
<td>0.046</td>
<td>8.7</td>
</tr>
<tr>
<td>B</td>
<td>1.534</td>
<td>0.149</td>
<td>9.7</td>
</tr>
<tr>
<td>C</td>
<td>5.905</td>
<td>0.457</td>
<td>7.7</td>
</tr>
</tbody>
</table>

TYPICAL CALIBRATOR CURVE

<table>
<thead>
<tr>
<th>Sample only.</th>
</tr>
</thead>
</table>

SAMPLE CALIBRATOR CURVE:

<table>
<thead>
<tr>
<th>Sample curve only.</th>
</tr>
</thead>
</table>

PERFORMANCE CHARACTERISTICS

SENSITIVITY

The limit of detection (LOD) was determined from the analysis of 64 samples of the blank and a low value sample and it was calculated as follows:

LOD = 3 × SD of blank + 3 × SD of low value sample

The limit of detection (LOD) was determined from the analysis of 64 samples of the blank and a low value sample and µB is calculated as follows:

µB = 3 × SD blank + 3 × SD low value sample

The following compounds were tested for cross-reactivity with Androstenedione to three patient serum samples. The results (in ng/mL) are tabulated below:

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<tr>
<th>Compound</th>
<th>Value (ng/mL)</th>
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<tbody>
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<td>DHEA</td>
<td>1.8</td>
</tr>
<tr>
<td>Testosterone</td>
<td>0.1</td>
</tr>
<tr>
<td>Estrone</td>
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</tr>
<tr>
<td>Estradiol</td>
<td>0.1</td>
</tr>
<tr>
<td>Progesterone</td>
<td>0.1</td>
</tr>
<tr>
<td>17α-Hydroxyprogesterone</td>
<td>0.1</td>
</tr>
<tr>
<td>5α-DHT</td>
<td>0.1</td>
</tr>
<tr>
<td>Cortisol</td>
<td>0.01</td>
</tr>
<tr>
<td>DHEA-S</td>
<td>0.01</td>
</tr>
</tbody>
</table>

SPECIFICITY (CROSS-REACTIVITY)

The following compounds were tested for cross-reactivity with androstenedione cross-reacting at 100%.

<table>
<thead>
<tr>
<th>Compound</th>
<th>% Cross Reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Androstenedione</td>
<td>100</td>
</tr>
<tr>
<td>DHEA</td>
<td>1.8</td>
</tr>
<tr>
<td>Testosterone</td>
<td>0.2</td>
</tr>
<tr>
<td>Estrone</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>Estradiol</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>Progesterone</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>17α-Hydroxyprogesterone</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>5α-DHT</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>Cortisol</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>DHEA-S</td>
<td>&lt; 0.01</td>
</tr>
</tbody>
</table>

EXPECTED NORMAL VALUES

As for all clinical assays each laboratory should conduct data and establish their own range of expected normal values.

<table>
<thead>
<tr>
<th>Group</th>
<th>Male Mean (ng/mL)</th>
<th>Female Mean (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td>20</td>
<td>2.0</td>
</tr>
<tr>
<td>Females</td>
<td>14</td>
<td>0.3–2.4</td>
</tr>
</tbody>
</table>