TESTOSTERONE SALIVA ELISA

Direct immunoenzymatic determination of Testosterone in saliva.

INTENDED USE
Eagle Biosciences Testosterone Saliva ELISA Assay Kit is a competitive immunoenzymatic colorimetric method for quantitative determination of Testosterone concentration in saliva. Testosterone Saliva ELISA kit is intended for research use only and not intended for diagnostic procedures.

1. CLINICAL SIGNIFICANCE
Testosterone (17β-Hydroxy-4-androstene-3-one) is a steroid hormone from the androgen group. In postpubertal males, testosterone is secreted primarily by the testes with only a small amount derived from peripheral conversion of androstenedione. In adult women over 50% of serum testosterone is derived from peripheral conversion of androstenedione secreted by the adrenal and ovary, with the remainder from direct secretion of testosterone by these glands. The level of testosterone in saliva (pg/mL) is significantly lower than levels in the general circulation (ng/mL).

Testosterone effects can be classified as virilizing and anabolic effects, although the distinction is somewhat artificial, as many of the effects can be considered both. 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, and after birth (usually at puberty) a deepening of the voice, growth of the beard and axillary hair (male secondary sex characteristics).

Testosterone levels decline gradually with age in men (andropause). The signs and symptoms are non-specific, and are generally associated with aging such as loss of muscle mass and bone density, decreased physical endurance, decreased memory ability and loss of libido. In females of all ages, elevated testosterone levels can be associated with a variety of virilizing conditions, including adrenal tumors and polycystic ovarian disease.

2. PRINCIPLE
In the Testosterone Saliva ELISA Assay Kit, the Testosterone (antigen) in the sample competes with the antigenic Testosterone conjugated with horseradish peroxidase (HRP) for binding to the limited number of antibodies anti Testosterone coated on the microplate (solid phase).

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 (H₂O₂) and the TMB Substrate and develops a blue color that changes into yellow when the Stop Solution (H₂SO₄) is added. The color intensity is inversely proportional to the Testosterone concentration in the sample. Testosterone concentration in the sample is calculated through a calibration curve.

3. REAGENTS, MATERIALS AND INSTRUMENTATION

3.1. Reagent and material supplied in the kit
1. Calibrators (5 vials, 1 mL each)
   - CAL0
   - CAL1
   - CAL2
   - CAL3
   - CAL4

2. Controls (2 vials, 1mL each)
   - Control A
   - Control B

Controls Concentration is indicted on the Certificate of Analysis.

3. Incubation Buffer (1 vial, 30 mL)
   Phosphate buffer pH 7.5, BSA 1 g/L

4. Conjugate (1 vial, 1 mL)
   Testosterone conjugated with Horseradish peroxidase (HRP)

5. Coated Microplate (1 breakable microplate)
   Anti Testosterone antibody absorbed on microplate

6. TMB Substrate (1 vial, 15 mL)
   H₂O₂-TMB 0.26 g/L (avoid any skin contact)
7. **Stop Solution** (1 vial, 15 mL)
   Sulphuric acid 0.15 mol/L *(avoid any skin contact)*
   
   8. **10X Conc. Wash Solution** (1 vial, 50 mL)
   Phosphate buffer 0.2M, pH 7.4
   
3.2. Reagents necessary not supplied
   Distilled water.

3.3. Auxiliary materials and instrumentation
   Microplates reader (450 nm, 620-630 nm)
   Automatic dispenser.
   Saliva Collection Device
   
   **Note**
   *Store all reagents at 2-8°C in the dark.*
   *Open the bag of reagent 5 (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 Testosterone ELISA Assay Kit is intended for research 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.
   - Material of animal igin used in the preparation of the kit has been obtained from animals certified as healthy and the bovine protein has been obtained from countries not infected by BSE, but these materials should be handled as potentially infectious.
   - Some reagents of the Testosterone Saliva ELISA Assay Kit contain small amounts of Proclin 300® 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/H₂O₂ to directed sunlight, metals or oxidants. Do not freeze the solution.
   - This Testosterone Saliva ELISA Assay Kit allows the determination of Testosterone from 10 pg/mL to 1000 pg/mL.
   - Testosterone can be invalidated if the patient 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 Testosterone Saliva 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 Testosterone Saliva ELISA Assay Kit components and specimens to reach room temperature (22-28°C) and mix well prior to use.
   - Do not interchange Testosterone Saliva ELISA Assay Kit components from different lots. The expiry date printed on box and vials labels 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.
   - The incomplete or inaccurate liquid removal from the wells could influence the assay precision and/or increase the background. To improve the performance of the kit on automatic systems is recommended to increase the number of washes.
   - 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 saliva.
   - Maximum precision is required for reconstitution and dispensation of reagents.
   - Plate readers measure vertically. Do not touch the bottom of the wells.

6. **PROCEDURE**

6.1. **Preparation of the Calibrators (C₀...C₄)**
   Before use, mix for 5 minutes with a rotating mixer.
   The Calibrators are ready to use and have the following concentration of Testosterone:

<table>
<thead>
<tr>
<th>pg/mL</th>
<th>C₀</th>
<th>C₁</th>
<th>C₂</th>
<th>C₃</th>
<th>C₄</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>10</td>
<td>50</td>
<td>200</td>
<td>1000</td>
</tr>
</tbody>
</table>

   For samples with concentration higher than 1000 pg/mL dilute the sample 1:2 with C₀.
   Once opened, the Calibrators are stable at 2-8°C for 6 months.
   For SI UNITS: pg/mL x 3.47 = pmol/L

6.2. **Preparation of Diluted Conjugate**
   Prepare immediately before use.
   Add 10 μL Conjugate (reagent 4) to 1.0 mL of Incubation Buffer (reagent 3). The volumes can be varied according to this proportion.
   Mix gently, for 5 minutes on a rotating shaker. Stable for 3 hours at room temperature (22-28°C).
6.3. Preparation of Wash Solution
Dilute the content of each vial of the "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 the complete dissolution of crystals; for greater accuracy, dilute the whole bottle of concentrated wash solution to 500 mL, taking care to transfer completely the crystals, then mix until crystals are completely dissolved.

6.4. Preparation of the Sample and Controls
The determination of Testosterone with this kit should be performed in saliva samples.
It is recommended to collect saliva samples with a centrifuge glass tube and a plastic straw, with the Eagle Biosciences Saliva Collection Device or with the “Salivette” (Sarstedt, Ref. 511534500). Other commercially available sample collector devices have not been tested.
The Controls are ready to use.

6.4.1. Method and Limitations
Collect saliva samples at the times indicated.
If no specific instructions have been given, saliva samples may be collected at any time; however the following should be noted:
a) If saliva collection is carried out in the morning ensure that this is carried out prior to brushing teeth
b) During the day allow 1 hour after a meal, oral intake of pharmaceutical drugs or tooth cleaning.
c) It is very important that a good clear sample is received – i.e. no contamination with food, lipstick, blood (bleeding gums) or other extraneous materials.

6.4.2. Saliva Processing Instructions with Saliva Collection Device Eagle Bioscience
1. Let the saliva flow down through the straw into the centrifuge glass tube.
2. Centrifuge the sample for 15 minutes at 3000 rpm
3. Store at – 20°C for at least 1 hour
4. Centrifuge again for 15 minutes at 3000 rpm
5. The saliva sample is now ready to be tested.
6. Store the sample at 2-8°C for one week or at – 20°C for longer time.

6.4.3. Saliva Processing Instructions with Salivette Sarstedt
1. Remove the swab from the suspended insert of the Salivette
2. Gently chewing the swab for 1 minute produces a sufficient quantity of saliva.
3. Replace the swab into the Salivette and firmly close the tube using the stopper.
4. Centrifuge the Salivette for 2 minutes at 1000g (rcf) for saliva generation.
5. Remove the insert complete with the swab from the centrifuge vessel and discard. The clear saliva is now ready for analysis (at least 1 mL of saliva should be recovered with this method).

6.5. Procedure
- Allow all reagents to reach room temperature (22-28°C). 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 (C₀-C₄), two for each Control, two for each sample, one for Blank.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Calibrator</th>
<th>Sample</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample/Controls</td>
<td>100 µL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calibrator C₀-C₄</td>
<td>100 µL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diluted Conjugate</td>
<td>100 µL</td>
<td>100 µL</td>
<td></td>
</tr>
</tbody>
</table>

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. 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.

7. QUALITY CONTROL
Each laboratory should assay controls at normal, high and low levels range of 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 (C₀-C₄) and of each sample.

8.2. Calibration curve
Plot the mean value of absorbance (Em) of the Calibrators (C₀-C₄) 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
As the values of salivary Testosterone have a circadian pattern we suggest to collect the samples at the same hour (8 A.M.).

The following values can be used as preliminary guideline until each laboratory established its own normal range.

<table>
<thead>
<tr>
<th></th>
<th>pg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>WOMEN:</td>
<td></td>
</tr>
<tr>
<td>normal</td>
<td>10 – 55</td>
</tr>
<tr>
<td>Hirsutism</td>
<td>25 – 85</td>
</tr>
<tr>
<td>(after treatment)</td>
<td>16 – 40</td>
</tr>
<tr>
<td>PCO</td>
<td>20 – 50</td>
</tr>
<tr>
<td>CHILDREN:</td>
<td>35 – 160</td>
</tr>
<tr>
<td>MEN:</td>
<td>50 – 210</td>
</tr>
<tr>
<td>Hipogonadism</td>
<td>10 – 80</td>
</tr>
</tbody>
</table>

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 Manufacturer 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 measurements (16x) of three different saliva samples in one assay. The within assay variability is ≤ 10.7%.

10.1.2. Inter Assay Variation
Between run variation was determined by replicate measurements (12x) of three different saliva samples in different lots of kit. The between assay variability is ≤ 13.2%.

10.2. Accuracy
The recovery of 250 - 500 pg/mL of Testosterone added to two saliva samples gave an average value (±SD) of 103.41% ± 8.92% with reference to the original concentrations.

The dilution test performed on 3 samples diluted up to 8 times gave an average value (±SD) of 99.63% ± 7.94%

10.3. Sensitivity
The lowest detectable concentration of Testosterone that can be distinguished from the Calibrator 0 is 3.98 pg/mL at the 95% confidence limit.

10.4. Specificity
The cross reaction of the antibody calculated at 50% according to Abraham are shown in the table:

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone</td>
<td>100%</td>
</tr>
<tr>
<td>Dihydrotestosterone</td>
<td>2.03%</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>0.01%</td>
</tr>
<tr>
<td>Andosterone</td>
<td>0.05%</td>
</tr>
<tr>
<td>DHEA-S</td>
<td>0.00%</td>
</tr>
<tr>
<td>Cortisol</td>
<td>0.01%</td>
</tr>
<tr>
<td>Cortisone</td>
<td>0.00%</td>
</tr>
<tr>
<td>17b Estradiol</td>
<td>0.16%</td>
</tr>
<tr>
<td>Estrone</td>
<td>0.01%</td>
</tr>
<tr>
<td>Prednisone</td>
<td>0.00%</td>
</tr>
</tbody>
</table>

10.5. Correlation
The new Eagle Biosciences Testosterone saliva ELISA kit was compared to the old Eagle Biosciences Testosterone saliva ELISA kit. 37 saliva samples (18 females, 19 males) were analysed. The linear regression curve was calculated:

\[ y = 0.7710 x – 12.29 \]

\[ r^2 = 0.884 \]

11. WASTE MANAGEMENT
Reagents must be disposed off in accordance with local regulations.

BIBLIOGRAPHY
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