The Testosterone ELISA Assay kit is intended for research use only and not to be used in diagnostic procedures.

1. CLINICAL SIGNIFICANCE
Testosterone (17β-OH-4-androstene-3-one) is a steroid hormone family of androgens mainly produced by the Leydig cells located in the testes and, minimally, by the ovaries and the adrenal cortex. It is also present in women who, compared to men, have a greater tendency to convert into estrogen this hormone.

In postpubertal males, testosterone is secreted primarily by the testes with only a small amount derived from peripheral conversion of androstenedione. In humans, in puberty, the development of the sexual organs (differentiation of the testes and the whole genital apparatus) and of secondary sexual characteristics, such as beard, hair distribution, the timbre of the voice and muscles. The testosterone, during puberty, is also involved on skeletal development, limiting the elongation of the long bones and avoiding, in this way, a disproportionate growth of the limbs. In adult humans, the levels of testosterone have a very important role as regards the sexuality, the musculoskeletal system, the vitality and good health (mainly understood as protection from metabolic diseases such as hypertension and diabetes mellitus); helps to ensure fertility, as it stimulates the maturation of sperm in the testes. Also influence the quality and quantity of sperm produced, since the seminal work on the streets and on the prostate, deputies to the production of sperm. Daily production of testosterone in men varies from 5 to 7 milligrams, but exceeded 30 years, tends to decrease annually by 1%. 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 majority of circulating testosterone is bound by SHBG and a smaller portion is bound by albumin. Only a small percentage (< 1%) exists in circulation as unbound or free testosterone.

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 ELISA Assay kit, the testosterone in the blood is bound to SHBG (60%) and in lower quantity to other proteins (for example albumin); the unbound Testosterone (< 1% of totalTestosterone) is known as “free Testosterone”. The chemical formulation of this assay allows to release completely the Testosterone from bound proteins; thus Diametra Testosterone kit allows to measure the concentration of total Testosterone (bound + free) in the sample. For the measurement of free Testosterone only, ELISA Diametra “Free Testosterone” kit is available.

Testosterone (antigen) in the sample competes with the antigenic Testosterone conjugated with horseradish peroxidase (HRP) present in the Conjugate for binding to the antibodies anti-testosterone coated on the microplates (solid phase). After the incubation, the bound/free separation is performed by a simple solid-phase washing.
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 colour 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. Reagents and materials supplied in the kit
1. Calibrators (5 vials, 1 mL each)
   - CAL0
   - CAL1
   - CAL2
   - CAL3
   - CAL4
   - REF DCE002/0206-0
   - REF DCE002/0207-0
   - REF DCE002/0208-0
   - REF DCE002/0209-0
   - REF DCE002/0210-0
2. Control (1 vial, 1 mL)
   - Control Concentration is Lot-specific and is indicated on the Certificate of Analysis
   - REF DCE045/0203-0
3. Conjugate (1 vial, 12 mL)
   - Testosterone conjugated with horseradish peroxidase (HRP)
   - REF DCE002/0202-0
4. Coated Microplate (1 breakable microplate)
   - Anti Testosterone antibody adsorbed on microplate
   - REF DCE002/0203-0
5. TMB Substrate (1 vial, 15 mL)
   - H₂O₂-TMB 0.26 g/L (avoid any skin contact)
   - REF DCE004-0
6. Stop Solution (1 vial, 15 mL)
   - Sulphuric acid 0.15 mol/L (avoid any skin contact)
   - REF DCE005-0
7. 10X Conc. Wash Solution (1 vial, 50 mL)
   - Phosphate buffer 0.2M, Proclin < 0.0015%
   - REF DCE054-0

3.2. Reagents necessary not supplied
- Distilled water
- Microplates reader (450 nm, 620-630 nm)

Notes
- Store all reagents at 2-8°C in the dark.
- Open the bag of reagent 4 (Coated Microplate) only when it is at room temperature and close it immediately after use; once opened, the microplate is stable until expiry date of the kit.

4. WARNINGS
- This Testosterone ELISA Assay Kit is intended for Research Use Only 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.
- Some reagents of this Testosterone 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 ELISA Assay Kit allows the determination of Testosterone from 0.2 ng/mL to 16.0 ng/mL.
- The clinical significance of the determination Testosterone can be invalidated if the sample 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 this Testosterone 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 ELISA Assay Kit components and specimens to reach room temperature (22-28°C) and mix well prior to use.
- Do not interchange Testosterone 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. To improve the performance of the kit on ELISA automatic systems, it is recommended to increase the number of washes.
- The incomplete or inaccurate liquid removal from the wells could influence the assay precision and/or increase the background.
- 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 sera.
- Maximum precision is required for reconstitution and dispensation of the reagents.
- Samples microbiologically contaminated, highly lipemic or haemolysed should not be used in the assay.
- Plate readers measure vertically. Do not touch the bottom of the wells.

6. PROCEDURE

6.1. Preparation of the Calibrators (C₀…C₄)
The Calibrators are ready for use and have the following concentration of Testosterone:

<table>
<thead>
<tr>
<th></th>
<th>C₀</th>
<th>C₁</th>
<th>C₂</th>
<th>C₃</th>
<th>C₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>ng/mL</td>
<td>0</td>
<td>0.2</td>
<td>1.0</td>
<td>4.0</td>
<td>16.0</td>
</tr>
</tbody>
</table>

Once opened, the Calibrators are stable six months at 2-8°C.

6.2. Preparation of the Conjugate
Ready to use. Mix gently for 5 minutes with a rotating mixer.
Once opened, it is stable six months at 2-8°C.

6.3. Preparation of the Sample
The determination of Testosterone can be performed in human plasma as well as in human serum. Store the sample at -20°C if the determination is not performed on the same day of the sample connection. Avoid repetitive freezing and thawing of samples. The Control is ready for use.

6.4. 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.

6.5. Procedure
- Allow all reagents to reach room temperature (22-28°C) for at least 30 minutes. 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>Sample/Control</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample/Control</td>
<td>25 µL</td>
<td></td>
</tr>
<tr>
<td>Calibrator</td>
<td>25 µL</td>
<td></td>
</tr>
<tr>
<td>C₀-C₄</td>
<td>100 µL</td>
<td>100 µL</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.

**Automatic washer:** in case you use an automatic washer, it is advised to do 6 washing steps.

<table>
<thead>
<tr>
<th>TMB Substrate</th>
<th>100 µL</th>
<th>100 µL</th>
<th>100 µL</th>
</tr>
</thead>
</table>

Incubate at room temperature (22-28°C) for 15 minutes in the dark.

<table>
<thead>
<tr>
<th>Stop Solution</th>
<th>100 µL</th>
<th>100 µL</th>
<th>100 µL</th>
</tr>
</thead>
</table>

Shake the microplate gently. Read the absorbance (E) at 450 nm against a reference wavelength of 620-630 nm or against Blank within 5 minutes.

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 absorbances (Em) for each point (C0-C4) of the calibration curve 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 ng/mL.

9. REFERENCE VALUES
The serum/plasma Testosterone reference values are:

<table>
<thead>
<tr>
<th></th>
<th>ng/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>WOMEN</td>
<td>0.2 – 1.2</td>
</tr>
<tr>
<td>CHILDREN</td>
<td>0.1 – 0.4</td>
</tr>
<tr>
<td>MEN</td>
<td>1.8 – 9.0</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 the measurements (16x) of three different control sera in one assay. The within assay variability is ≤ 5.8%.

10.1.2. Inter Assay Variation
Between run variation was determined by replicate the measurements (16x) of three different control sera in different lots. The between assay variability is ≤ 10.5%.

10.2. 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>16%</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>0.8%</td>
</tr>
<tr>
<td>Androsterone</td>
<td>0%</td>
</tr>
<tr>
<td>DHEA-S</td>
<td>0%</td>
</tr>
<tr>
<td>Cortisol</td>
<td>0%</td>
</tr>
<tr>
<td>Cortisone</td>
<td>0%</td>
</tr>
<tr>
<td>17 α Estradiol</td>
<td>0%</td>
</tr>
<tr>
<td>Estrone</td>
<td>0%</td>
</tr>
<tr>
<td>Prednisone</td>
<td>0%</td>
</tr>
</tbody>
</table>

10.3. Accuracy
The recovery of 0.2 – 1.0 – 4.0 – 16 ng/mL of Testosterone added to sample gave an average value of 105.5% with reference to the original concentrations.

10.4. Sensitivity
The lowest detectable concentration of Testosterone that can be distinguished from the Calibrator 0 is 0.07 ng/mL at the 95% confidence limit.

10.5. Correlation with RIA
Diametra Testosterone kit was compared to another commercially available Testosterone assay. Serum samples of 25 females and 27 males were analysed according in both test systems. The linear regression curve was calculated: (Testost. Diametra) = 0.97*(Testost. RIA) + 0.13 \( r^2 = 0.99 \)

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