Testosterone ELISA Kit

Enzyme Immunoassay for the quantitative determination of free active Testosterone in saliva

Catalog number: ARG80867

Package: 96 wells

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MANUFACTURED BY:

Arigo Biolaboratories Corporation

Address: No. 22, Ln. 227, Gongyuan Rd., Hsinchu City 300, Taiwan

Phone: +886 (3) 562 1738

Fax: +886 (3) 561 3008

Email: info@arigobio.com

INTRODUCTION

At present, the majority of steroid hormone determinations are conducted from serum samples, even if results in the low or very low concentration range are expected, for example, in elderly patients. This is a real challenge for any diagnostic laboratory as shown by Taieb et al in 2003 and others. Recently there has been an official position statement of the Endocrine Society stating that reliable Testosterone measurements in serum either need an extraction step or have to be done by chromatographic methods like Tandem MS or GCMS. There now is sufficient evidence that the commercial Testosterone assays are unable to quantify low concentrations in a reliable way. Another major problem associated with the measurement of free hormone levels from serum is the episodic secretion pattern of steroid hormones. Even in 1973 it could be shown that steroid secretion shows a significant episodic pattern. Nevertheless, the majority of the determinations are still made from just one serum sample, resulting in non-reproducible values due to the biological variation. In general, serum measurements can only give the total steroid hormone concentration, whereas saliva testing results in the measurement of the free active hormone fraction. So far, all attempts for a direct quantification of free Testosterone in serum or plasma samples by commercial immunoassays have failed. Taking into consideration the above mentioned drawbacks of the current analytical procedures, salivary testing seems to be a reliable alternative. It has been shown that the measurement of free salivary Testosterone gives clinically valid results even in the low concentration range.

PRINCIPLE OF THE ASSAY

This assay employs the competitive enzyme immunoassay technique. A highly specific Testosterone antibody has been pre-coated onto a microtiter plate. Testosterone containing samples or standards and a Testosterone-HRP conjugate are given into the wells of the microtiter plate. Enzyme labeled and free Testosterone compete for the antibody binding sites. After incubation at room temperature, the wells are washed with diluted washing solution to remove unbound material. A substrate solution is added and incubated, resulting in the development of a blue color. The color development is inhibited by the addition of a stop solution, and the color turns yellow. The yellow color is measured at 450 nm. The concentration of Testosterone is indirectly proportional to the color intensity of the test sample.

MATERIALS PROVIDED & STORAGE INFORMATION

Store the unopened kit at 2-8 °C. Use the kit before expiration date.

Component	Quantity	Storage information
Antibody-coated microplate	12 strips x 8-well	4°C
HRP-Testosterone Conjugate	12 ml	4°C
Standard 0	3 ml	4°C
Standards 1-5 (10, 30, 100, 300, 1000 pg/ml)	5 X 1 ml (ready to use)	4°C
Control Low (Acc. Range: 35-73 pg/ml)	1 ml (ready to use)	4°C
Control High (Acc. Range: 127-265 pg/ml)	1 ml (ready to use)	4°C
10X Wash Buffer	50 ml	4°C
TMB substrate	22 ml (ready to use)	4°C (Protect from light)
STOP solution	7 ml (ready to use)	4°C

MATERIALS REQUIRED BUT NOT PROVIDED

- Microplate reader capable of measuring absorbance at 450nm
- Microtiter plate shaker (shaking amplitude 3 mm; approx. 600 rpm)
- Pipettes and pipette tips
- Deionized or distilled water
- Automated microplate washer (optional)

TECHNICAL HINTS AND PRECAUTIONS

- Wear protective gloves, clothing, eye, and face protection especially while handling blood or body fluid samples.
- Store the kit at 2-8°C at all times. After first opening the reagents are stable for 30 days if used and stored properly.
- Do not use reagents beyond expiry date as shown on the kit labels.
- If crystals are observed in the 10X Wash buffer, warm to RT (or 37°C) until the crystals are completely dissolved.
- Ensure complete reconstitution and dilution of reagents prior to use.
- It is highly recommended that the standards, samples and controls be assayed in duplicates.
- Allow the reagents and the required number of wells to reach room temperature (21-26°C) before starting the test. Temperature will affect the absorbance readings of the assay. However, values for the patient samples will not be affected.
- All reagents must be mixed without foaming.
- Mix the contents of the microplate wells thoroughly to ensure good test

results. Do not reuse microwells.

- Do not let wells dry during assay; add reagents immediately after completing the rinsing steps.
- Once the test has been started, all steps should be completed without interruption.
- Absorbance is a function of the incubation time and temperature. Before starting the assay, it is recommended that all reagents are ready, caps removed, all needed wells secured in holder, etc. This will ensure equal elapsed time for each pipetting step without interruption.
- Use reservoirs only for single reagents. This especially applies to the substrate reservoirs. Using a reservoir for dispensing a substrate solution that had previously been used for the conjugate solution may turn solution colored. Do not pour reagents back into vials as reagent contamination may occur.
- As a general rule the enzymatic reaction is linearly proportional to time and temperature.
- Respect the incubation times as stated in this instructions for use.
- All indicated volumes have to be performed according to the protocol. Optimal test results are only obtained when using calibrated pipettes and microtiterplate readers.
- Change pipette tips between the addition of different reagent or samples.
- Samples contain azide cannot be assayed.

SAMPLE COLLECTION & STORAGE INFORMATION

The saliva samples should be completely colorless. Even the slightest red color shows blood contamination. Such blood contamination will give falsely elevated concentration values. In case of visible blood contamination the patient should discard the sample, rinse the sampling device with water, wait for 10 minutes and take a new sample. Samples containing sodium azide should not be used in the assay.

Collection –

1. For the correct collection of saliva we are recommending to only use appropriate devices made from ultrapure polypropylene. Do not use any PE devices or Salivates for sampling; this in most cases will result in significant interferences. Glass tubes can be used as well, but in this case special attention is necessary for excluding any interference caused by the stopper.

2. As the Testosterone secretion in saliva as well as in serum shows an obvious episodic secretion pattern it is important to care for a proper timing of the sampling. In order to avoid arbitrary results we are recommending collecting 5 samples within a period of 2 hours (multiple sampling) preferably in the early morning of a normal day directly after waking up. As food might contain significant amounts of steroid hormones samples preferably should be taken while fasting. If fasting should be a problem the collection period should be timed just before lunch or before dinner. In the early morning Testosterone levels of males are significantly higher compared to those ones during the day. The Testosterone concentration in the morning is roughly twice as high compared to the evening concentration.

Do not chew anything during the sampling period. Any pressure to the teeth may result in falsely elevated measurements due to an elevated content of gingival liquid in the saliva sample.

Storage and Preparation-

Saliva samples in general are stable at ambient temperature for up to seven days. Therefore mailing of such samples by ordinary mail without cooling will not create a problem. Storage at 4°C can be done for a period of up to four weeks. Whenever possible samples preferable should be kept at a temperature of-20°C. Even repeated thawing and freezing is no problem. Each sample has to be frozen, thawed, and centrifuged at least once anyhow in order to separate the mucins by centrifugation. Upon arrival of the samples in the lab the samples have to stay in the deep freeze at least overnight. Next morning the frozen samples are warmed up to room temperature and mixed carefully. Then the samples have to be centrifuged for 5 to 10 minutes. Now the clear colorless supernatant is easy to pipette. If the sample should show even a slightly reddish color it should be discarded. Otherwise the value most probably will be falsely elevated. Due to the episodic variation of steroid secretion we highly recommend the strategy of multiple sampling. If such a set of multiple samples have to be tested the lab (after at least one freezing, thawing, and centrifugation cycle) has to mix the aliquots of the 5 single samples in a separate sampling device and perform the testing from this mixture.

REAGENT PREPARATION

- 1X Wash buffer: Dilute 10X Wash buffer into distilled water to yield 1X Wash buffer. (E.g. 50 ml of 10X Wash buffer + 450 ml of distilled water to a final volume of 500 ml.) The diluted 1X Wash buffer is stable for 3 months at room temperature.
- Sample: If in an initial assay, a specimen is found to contain more than the highest standard (1000 pg/ml), the specimens can be diluted with Standard 0 and re-assayed as described in Assay Procedure.

  For the calculation of the concentrations this dilution factor has to be taken into account.

  Example:

  a) Dilution 1:10: 10 µl saliva + 90 µl Standard 0 (mix thoroughly)

  b) Dilution 1:100: 10 µl of dilution a + 90 µl Standard 0 (mix thoroughly).

ASSAY PROCEDURE

All materials should be equilibrated to room temperature (RT, 21-26°C) before use. All reagents must be mixed without foaming. Standards, samples and controls should be assayed in duplicates. Each run must include a standard curve.

1. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal it.
2. Add **100 µl** of **Standards, Controls and Samples** in duplicate into the appropriate wells.
3. Add **100 µl** of **HRP-Testosterone Conjugate** into each well. Incubate for **60 minutes** at **RT** on a microplate shaker at ~600 rpm. (Optimal reaction in this assay is markedly dependent on shaking of the microplate!)
4. Aspirate each well and wash, repeating the process 3 times for a **total 4 washes.** Wash by filling each well with **1X Wash Buffer (300 µl)** using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating, decanting or blotting against clean paper towels.
5. Add **200 µl** of **TMB substrate** to each well. Incubate for **30 minutes at room temperature** in dark.
6. Add **50 µl** of **Stop Solution** to each well.
7. Read the OD with a microplate reader at 450 nm immediately. It is recommended read the absorbance within 15 min after adding Stop solution.

CALCULATION OF RESULTS

1. Calculate the average absorbance values for each set of standards, controls and patient samples.

2. Using linear graph paper, construct a standard curve by plotting the mean absorbance obtained from each standard against its concentration with absorbance value on the vertical (Y) axis and concentration on the horizontal (X) axis.

3. Using the mean absorbance value for each sample determine the corresponding concentration from the standard curve.

4. Automated method: The results in the IFU have been calculated automatically using a 4 PL (4 Parameter Logistics) curve fit. 4 Parameter Logistics is the preferred method. Other data reduction functions may give slightly different results. The diluted samples must be further converted by the appropriate dilution factor according to the sample preparation procedure as described above.

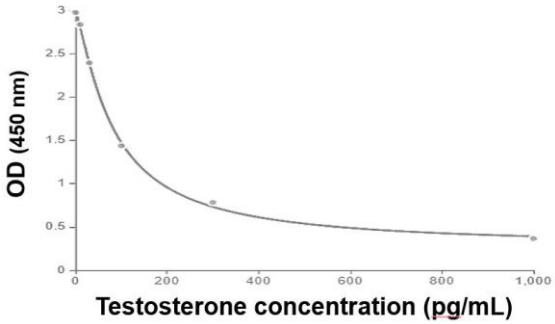
5. arigo provides GainData®, an in-house development ELISA data calculator, for ELISA data result analysis. Please refer our GainData® website for details. (https://www.arigobio.com/elisa-analysis)

6. If the samples have been diluted, the concentration read from the standard curve must be further converted by the appropriate dilution factor according to the sample preparation procedure as described above.

7. Conversion to SI units: Testosterone (pg/ml) x 3.47 = pmol/l.

EXAMPLE OF TYPICAL STANDARD CURVE

The following data is for demonstration only and cannot be used in place of data generations at the time of assay.



QUALITY ASSURANCE

Sensitivity

The analytical sensitivity was calculated from the mean plus two standard deviations of twenty (20) replicate analyses of Standard 0 is 2.2 pg/ml.

Assay dynamic range

The range of the assay is between 10 – 1000 pg/ml.

Intra-assay and inter-assay precision

The CV value of intra-assay precision was 7.47% and the CV value of inter-assay precision was 9.13%.

Recovery

80.4-116%

Linearity

74-117%

Specificity

The following substances were tested for cross reactivity of the assay:

Substance	Cross reactivity (%)
Testosterone	100
5α-Dihydrotestosterone	23.3
Androstenedione	32.2
Androsteron	< 0.1
5α-Androstane	< 0.1
5ß-Androstane-3α,17ß-diole	< 0.1
Corticosterone	< 0.1
11-Desoxycorticosterone	< 0.1
Dexamethasone	< 0.1
Estradiol	< 0.1
Progesterone	< 0.1
17α-Hydroxyprogesterone	< 0.1
Cortisol	< 0.1
11-Desoxycortisol	< 0.1
Cortison	< 0.1
Estrone	< 0.1
Pregenolone	< 0.1
Prednisone	< 0.1
Prednisolon	< 0.1
Prednisone	< 0.1
Danazol	< 0.1

Interfering Substances

Blood contamination in saliva samples will affect results, and usually can be seen by eye.

Drug Interferences

Any medication (cream, oil, pill, etc.) containing testosterone of course will significantly influence the measurement of this analytic.