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LABOR DIAGNOSTIKA NORD GmbH & Co.KG | Am Eichenhain 1 | 48531 Nordhorn | Germany | Tel. +49 5921 8197-0 | Fax +49 5921 8197-222 | info@ldn.de | www.ldn.de

Instructions for use

Testosterone Saliva ELISA Free

REF SA E-6100R

2°C–8°C

Σ 96

RUO For research use only – Not for use in diagnostic procedures

TESTOSTERONE FREE IN SALIVA ELISA

1. INTRODUCTION

1.1 Intended Use

An Enzyme Immunoassay for the quantitative measurement of free active testosterone in saliva.
Measurement of testosterone is used in research of disorders involving the male sex hormones (androgens), including primary and secondary hypogonadism, delayed or precocious puberty, impotence in males and in females hirsutism (excessive hair) and virilization (masculinization) due to tumors, polycystic ovaries, and adrenogenital syndromes. It is intended only for research use.

1.2 Summary and explanation

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 laboratory as shown by Taieb et al in 2003 [10] and others [9]. Recently there has been an official position statement of the Endocrine Society[14] 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[1] 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 [3,5].
So far, all attempts for a direct quantification of free Testosterone in serum or plasma samples by commercial immunoassays have failed [7].
Taking into consideration the above mentioned drawbacks of the current analytical procedures, salivary testing seems to be a reliable alternative. It has been shown in the literature [3,5,13,15] that the measurement of free salivary Testosterone gives valid results even in the low concentration range. In salivary testing it is easy to compensate for the episodic secretion pattern provided multiple sampling is done (preferably 5 samples within 2 hours). The measurement of free Testosterone is done with a mixture of these 5 samples. In contrast to this, measurements from just one single saliva sample always will give arbitrary results (like in serum).

2. PRINCIPLE

The **Testosterone free in Saliva ELISA** Kit is a solid phase enzyme-linked immunosorbent assay (ELISA), based on the principle of competitive binding. An unknown amount of free testosterone present in the sample and a defined amount of testosterone conjugated to horseradish peroxidase compete for the binding sites of rabbit polyclonal testosterone antiserum coated to the wells of a microplate. After one-hour incubation on a shaker the microplate is washed four times. After addition of the substrate solution the concentration of testosterone is inversely proportional to the optical density measured.

3. WARNINGS AND PRECAUTIONS

1. This kit is for research use only.
2. Before starting the assay, read the instructions completely and carefully. Use the valid version of the package insert provided with the kit. Be sure that everything is understood.
3. The microplate contains snap-off strips. Unused wells must be stored at 2 °C to 8 °C in the sealed foil pouch and used in the frame provided.
4. Pipetting of samples and reagents must be done as quickly as possible and in the same sequence for each step.
5. 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.
6. Mix the contents of the microplate wells thoroughly to ensure good test results. Do not reuse microwells.
7. Do not let wells dry during assay; add reagents immediately after completing the rinsing steps.
8. Allow the reagents to reach room temperature (21-26°C) before starting the test. Temperature will affect the absorbance readings of the assay. However, values for the samples will not be affected.
9. Never pipet by mouth and avoid contact of reagents and specimens with skin and mucous membranes.
10. Do not smoke, eat, drink or apply cosmetics in areas where specimens or kit reagents are handled.

11. Wear disposable latex gloves when handling specimens and reagents. Microbial contamination of reagents or specimens may give false results.
12. Handling should be done in accordance with the procedures defined by an appropriate national biohazard safety guideline or regulation.
13. Do not use reagents beyond expiry date as shown on the kit labels.
14. All indicated volumes have to be performed according to the protocol. Optimal test results are only obtained when using calibrated pipettes and microtiterplate readers.
15. Do not mix or use components from kits with different lot numbers. It is advised not to exchange wells of different plates even of the same lot. The kits may have been shipped or stored under different conditions and the binding characteristics of the plates may result slightly different.
16. Avoid contact with Stop Solution. It may cause skin irritation and burns.
17. Chemicals and prepared or used reagents have to be treated as hazardous waste according to the national biohazard safety guideline or regulation.
18. For information please refer to Material Safety Data Sheets. Safety Data Sheets for this product are available upon request directly from the manufacturer.

4. REAGENTS PROVIDED

4.1 Reagents provided

Standards and Controls

	Cat. no.	Standard	Concentration	Volume / Vial
STANDARD A	**SA E-6101**	Standard A (0)	0 pg/ml	3 ml
STANDARD B	**SA E-6102**	Standard B (1)	10 pg/ml	1 ml
STANDARD C	**SA E-6103**	Standard C (2)	30 pg/ml	1 ml
STANDARD D	**SA E-6104**	Standard D (3)	100 pg/ml	1 ml
STANDARD E	**SA E-6105**	Standard E (4)	300 pg/ml	1 ml
STANDARD F	**SA E-6106**	Standard F (5)	1000 pg/ml	1 ml
CONTROL 1	**SA E-6151**	Control low	Please refer to QC-Datasheet	1 ml
CONTROL 2	**SA E-6152**	Control high		1 ml

Conversion: Testosterone (pg/ml) x 3.47 = pmol/l

TLT 96 **SA E-6131 Coated Microplate**

12 x 8 (break apart) strips with 96 wells;

Wells coated with an anti-Testosterone antibody (rabbit polyclonal antibody).

CONJUGATE **SA E-6140 Enzyme Conjugate**

1 vial, 12 ml, ready to use;

Testosterone conjugated to horseradish peroxidase

SUBSTRATE **AR E-0055 Substrate Solution**

1 vial, 22 ml, ready to use;

contains tetramethylbenzidine (TMB)

Hazards identification:

H360D May damage the unborn child.

STOP-SOLN **AR E-0080 Stop Solution**

1 vial, 7 ml, ready to use;

contains 2 N Hydrochloric Acid solution

Hazards identification:

H290 May be corrosive to metals.
H314 Causes severe skin burns and eye damage.
H335 May cause respiratory irritation.

WASH-CONC 10x **AR E-0030 Wash Solution**

1 vial, 50 ml (10X concentrated);

see „Preparation of Reagents".

Note: Additional *Standard A* for sample dilution is available upon request.

4.2 Materials required but not provided

- Microcentrifuge
- A microtiter plate reader capable for endpoint measurement at 450 nm
- Microplate mixer operating more than 600 rpm
- Vortex mixer
- Calibrated variable precision micropipettes (50 µl, 100 µl, 200 µl).
- Absorbent paper.
- Distilled or deionized water
- Timer
- Semi logarithmic graph paper or software for data reduction

4.3 Storage conditions

When stored at 2°C to 8°C unopened reagents will be stable until expiration date. Do not use reagents beyond this date. Opened reagents must be stored at 2°-8°C. After first opening the reagents are stable for 30 days if used and stored properly.
Microtiter wells must be stored at 2°C to 8°C. Take care that the foil bag is sealed tightly.

4.4 Reagent preparation

Allow the reagents and the required number of wells to reach room temperature (21-26°C) before starting the test.

***Wash Solution*:**
Dilute 50 ml of 10X concentrated Wash Solution with 450 ml deionized water to a final volume of 500 ml.
The diluted Wash Solution is stable for at least 3 months at room temperature.

4.5 Disposal of the kits

The disposal of the kit must be made according to the national regulations. Special information for this product is given in the Material Safety Data Sheet.

4.6 Damaged test kits

In case of any severe damage of the test kit or components, the manufacturer have to be informed in writing within one week after receiving the kit. Severely damaged single components should not be used for a test run. They have to be stored until a final solution has been found. After this, they should be disposed according to the official regulations.

5. SPECIMEN

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

5.1 Specimen Collection

For the correct collection of saliva we are recommending to use appropriate devices made from ultra-pure polypropylene. Do not use any PE devices for sampling to avoid significant interferences. Glass tubes can be used as well, but in this case special attention is necessary for excluding any interference caused by the stoppers. For more details please contact the manufacturer. 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 to collect 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.

5.2 Specimen 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 any problem. Storage at 4°C can be done for a period of up to one month. Whenever possible, samples should preferably be kept at a temperature of -20°C. Even repeated thawing and freezing is not a 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 at the lab the samples have to kept frozen at least overnight. Next morning the samples are thawed and mixed carefully. The samples have to be centrifuged for 5 to 10 minutes. The clear colorless supernatant is easy to pipette. If the sample should show even a slighty red color it should be discarded. Otherwise the value most probably will be falsely elevated. Due to the episodic variations of the steroid secretion we highly recommend the strategy of multiple sampling. If such a set of multiple samples has to be tested the staff of lab (after at least one freezing, thawing, and centrifugation cycle) should mix aliquots of the 5 single samples and perform the determination using the mixture.

5.3 Specimen Dilution

Samples expected to contain testosterone concentrations higher than the highest standard (1000 pg/ml) should be diluted with the standard A before assay. The additional dilution step has to be taken into account for the calculation of the result.

Example:

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

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

6. ASSAY PROCEDURE

6.1 General Remarks

- All reagents and specimens must be allowed to come to room temperature before use. All reagents must be mixed without foaming.
- Once the test has been started, all steps should be completed without interruption.
- Use new disposal plastic pipette tips for each standard, control or sample in order to avoid cross contamination.
- 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.
- 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.

6.2 Assay procedure

Each run must include a standard curve.

1. Prepare a sufficient number of microplate wells to accommodate standards, controls and samples.
2. Dispense **100 µl** of each ***Standard*, *Control*** **and sample** with new disposable tips into appropriate wells
3. Dispense **100 µl** of **Enzyme Conjugate** into each well.
4. Incubate for **60 minutes** at room temperature on a Microplate mixer (≥ 600 rpm).
   Important Note:
   Optimal reaction in this assay is markedly dependent on shaking of the microplate!
5. Discard the content of the wells and rinse the wells **4 times** with diluted Wash Solution (300 µl per well). Remove as much Wash Solution as possible by beating the microplate on absorbent paper.
6. Add **200 µl** of **Substrate Solution** to each well.
7. Incubate for **30 minutes** in the dark.
8. Stop the reaction by adding **50 µl** of **Stop Solution** to each well.
9. Determine the absorbance of each well at 450 nm. It is recommended to read the wells within 15 minutes.

6.3 Calculation of results

1. Calculate the average absorbance values for each set of standards, controls and patient samples.
2. 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 this IFU have been calculated automatically using a 4 PL (4 parameters Logistics) curve fit. 4 Parameters Logistics is the preferred calculation method. Other data reduction functions may give slightly different results.
5. The concentration of the samples can be determined directly from this standard curve. Samples with concentrations higher than that of the highest standard have to be further diluted. For the calculation of the concentrations, this dilution factor has to be taken into account.

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

6.3.1 Example of Typical Standard Curve

Following data are intended for illustration only and should not be used to calculate results from another run.

Standard	Optical Units (450 nm)
Standard A (0 pg/ml)	2.267
Standard B (10 pg/ml)	2.040
Standard C (30 pg/ml)	1.721
Standard D (100 pg/ml)	1.019
Standard E (300 pg/ml)	0.592
Standard F (1000 pg/ml)	0.299

7. EXPECTED NORMAL VALUES

In order to determine the normal range of salivary Testosterone, saliva samples from children, adult male and adult female apparently healthy subjects were collected in the morning and analyzed using the Testosterone free in Saliva ELISA kit. The following ranges are calculated with the results of this study.

The concentrations are given in pg/ml.

	Men ♂			Women ♀		
Age Group Years	5th-95th Percentile [pg/ml]	Median [pg/ml]	n	5th-95th Percentile [pg/ml]	Median [pg/ml]	n
15 – 55	33.6 – 205.0	90.0	83	11.6 – 88.1	33.8	538
>55	25.1 – 140.7	68.3	42	9.3 – 83.0	27.2	137

	Children		
Age Group Years	5th-95th Percentile [pg/ml]	Median [pg/ml]	n
≤ 11	5.8 – 45.3	11.5	8

8. QUALITY CONTROL

Good laboratory practice requires that controls should be run with each standard curve. A statistically significant number of controls should be assayed to establish mean values and acceptable ranges to assure proper performance.
It is recommended to use control samples according to national regulations. The use of control samples is advised to assure the day to day validity of results. Use controls at both normal and pathological levels.
The controls and the corresponding results of the QC-Laboratory are stated in the QC certificate added to the kit. The values and ranges stated at the QC sheet always refer to the current kit lot and should be used for direct comparison of the results.
It is also recommended to make use of national or international Quality Assessment programs in order to ensure the accuracy of the results.
Employ appropriate statistical methods for analyzing control values and trends. If the results of the assay do not fit to the established acceptable ranges of control materials results should be considered invalid.
In this case, please check the following technical areas: Pipetting and timing devices; photometer, expiration dates of reagents, storage and incubation conditions, aspiration and washing methods.

9. PERFORMANCE CHARACTERISTICS

9.1 Analytical Sensitivity

The lowest analytical detectable level of testosterone that can be distinguished from the Standard A is 2.2 pg/ml at the 2SD confidence limit.

9.2 Specificity (Cross Reactivity)

The following materials have been evaluated for cross reactivity. The percentage indicates cross reactivity at 50% displacement compared to Testosterone.

Steroid	% Cross reaction
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%

9.3 Assay Dynamic range

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

9.4 Reproducibility

9.4.1 Intra-Assay

The intra-assay variation was determined by 20 replicate measurements of 3 saliva samples within one run. The within-assay variability is shown below:

	Sample 1	Sample 2	Sample 3
Mean (pg/ml)	61.0	90.7	216.3
SD	5.92	6.57	11.92
CV (%)	9.7	7.2	5.5
n =	20	20	20

9.4.2 Inter-Assay

The inter-assay (between-run) variation was determined by duplicate measurements of 3 saliva samples over 10 days.

	Sample 1	Sample 2	Sample 3
Mean (pg/ml)	53.4	74.6	288.3
SD	10.42	5.90	27.74
CV (%)	9.9	7.9	9.6
n =	11	11	11

9.5 Recovery

Using the Standard Matrix six spiking solutions were prepared (A = 2000 pg/ml, B = 4000 pg/ml, C = 6000 pg/ml, D = 500 pg/ml, E = 1000 pg/ml and F = 2000 pg/ml). A 25 µl aliquot of each solution was spiked into 475 µl of different salivas, for a spiking ratio of 1 to 20, leaving the saliva matrix of the spiked samples relatively intact. All samples were then measured by Salivary Testosterone procedure. To calculate expected values 95% of the unspiked values were added to 5% of the spiking solution concentrations.

Sample	Measured (pg/ml)	Expected (pg/ml)	Recovery (%)
1	61.8	-	-
	153.3	158.7	96.6
	259.0	258.7	100.1
	365.0	358.7	101.8
2	74.0	-	-
	164.6	170.3	96.6
	270.4	270.3	100.0
	345.4	370.3	93.3
3	30.9	-	-
	49.4	54.3	91.0
	76.6	79.3	96.6
	109.0	129.3	84.3

Sample	Measured (pg/ml)	Expected (pg/ml)	Recovery (%)
4	87.3	-	-
	105.8	107.9	98.0
	125.8	132.9	94.7
	195.6	182.9	106.9
5	71.1	-	-
	85.6	92.5	92.5
	94.5	117.5	80.4
	154.9	167.5	92.5
6	74.7	-	-
	87.9	95.9	91.6
	140.3	120.9	116.0
	168.4	170.9	98.5

9.6 Linearity

Six saliva samples containing different amounts of analyte were serially diluted with zero standard (A) and assayed with the ELISA. Four native samples were serially diluted, and two samples were spiked with testosterone and then serially diluted up to 1:8. The percentage recovery was calculated by comparing the expected and measured values for testosterone.

Saliva	Dilution	Observed 0	Expected (E)	O/E %
1	native	224	-	-
	1 in 2	118	112	105%
	1 in 4	51	56	91%
	1 in 8	27	28	96%
2	native	205	-	-
	1 in 2	110	103	107%
	1 in 4	50	51	98%
	1 in 8	27	26	104%
3	native	106.7	-	-
	1 in 2	55.6	53.4	104%
	1 in 4	27,7	26,7	104%
	1 in 8	11,9	13,3	89%
4	native	66.6	-	-
	1 in 2	28,9	33.3	87%
	1 in 4	12,3	16,70	74%
	1 in 8	6,9	8,30	83%
5	native	91.1	-	-
	1 in 2	51,1	45.6	112%
	1 in 4	26,3	22,8	115%
	1 in 8	12,9	11,4	113%
6	native	133.7	-	-
	1 in 2	62.6	66,9	94%
	1 in 4	33.0	33,4	99%
	1 in 8	19,5	16,7	117%

10. LIMITATIONS OF PROCEDURE

Reliable and reproducible results will be obtained when the assay procedure is performed with a complete understanding of the package insert instruction and with adherence to good laboratory practice.
Any improper handling of samples or modification of this test might influence the results.

10.1 Interfering Substances

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

10.2 Drug Interferences

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

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Symbols:

	Storage temperature		Manufacturer		Contains sufficient for <n> tests
	Expiry date	LOT	Batch code		
	Consult instructions for use	CONT	Content		
	Caution	REF	Catalogue number	RUO	For research use only!