DRG® Salivary Testosterone (SLV-3013)

Revised 12 Jan. 2008 (Vers. 8.1) **IVD**

INTRODUCTION

Intended Use

An Enzyme Immunoassay for the *in vitro diagnostic* quantitative measurement of free active testosterone in saliva.

Measurement of testosterone is used in the diagnosis and treatment 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.

Summary and Explanation

Testosterone (17β-hydroxy-4-androstene-3-one) is a C19 steroid with an unsaturated bond between C-4 and C-5, a ketone group in C-3 and a hydroxyl group in the β position at C-17. This steroid hormone has a molecular weight of 288.4 daltons. Testosterone is the most important androgen secreted into the blood. In males, primarily the Leydig cells of the testis secrete testosterone; in females approximately 50% of circulating testosterone is derived from peripheral conversion of androstenedione, approximately 25% from the ovary and 25% from the adrenal glands.
Testosterone is responsible for the development of secondary male sex characteristics and its measurements are helpful in evaluating the hypogonadal status (1-4). In women high levels of testosterone are generally found in hirsutism and virilisation, polycystic ovaries, ovarian tumors, adrenal tumors and adrenal hyperplasia (5-7). In men high levels of testosterone are associated with hypothalamic-pituitary-unit dysfunction, testicular tumors, congenital adrenal hyperplasia and prostate cancer. Low levels of testosterone are encountered in male patients with the following diseases: Klinefelter´s syndrome. Hypopituitarism, testicular feminization, orchidectomy, cryptorchidism, enzymatic defects and some autoimmune diseases (8).

PRINCIPLE

The **DRG Salivary Testosterone ELISA** Kit is based on the competition principle and the microplate separation. An unknown amount of free testosterone present in the sample and a fixed amount of testosterone conjugated with horseradish peroxidase compete for the binding sites of mouse monoclonal testosterone antiserum coated onto the wells. After one-hour incubation the microplate is washed to stop the competition reaction. After addition of the substrate solution the concentration of testosterone is inversely proportional to the optical density measured.

WARNINGS AND PRECAUTIONS

1. For in-vitro diagnostic use only. For professional 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. Do not mix reagents of different lots. Do not use expired reagents.
4. The microplate contains snap-off strips. Unused wells must be stored at 2 - 8°C in the sealed foil pouch and used in the frame provided.
5. Avoid contact with Stop Solution, 0.5M H_2SO_4. It may cause skin irritation and burns.
6. Pipetting of samples and reagents must be done as quickly as possible and in the same sequence for each step.
7. Use separate pipette tips for each sample, control and reagent to avoid cross contamination.

8. 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.
9. Mix the contents of the microplate wells thoroughly to ensure good test results. Do not reuse microwells.
10. Do not let wells dry during assay; add reagents immediately after completing the rinsing steps.
11. Some reagents contain Proclin, BND and MIT as preservatives. In case of contact with eyes or skin, flush immediately with water.
12. 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 patient samples will not be affected.
13. TMB substrate has an irritant effect on skin and mucosa. In case of possible contact, wash eyes with an abundant volume of water and skin with soap and abundant water. Wash contaminated objects before reusing them. If inhaled, take the person to open air.

REAGENTS

Reagents provided

1. ***Microtiterwells***, 12x8 (break apart) strips, 96 wells;
   Wells coated with a anti-Testosterone antibody (monoclonal).
2. ***Standard (Standard 0-6)*****,** 7 vials, 1 mL each, ready to use;
   Concentrations: 0.0 – 10 – 50 – 100 – 500 – 1000 – 5000 pg/mL
   Conversion: Testosterone (pg/mL) x 3.47 = pmol/L
   * contain 0.03% Proclin 300, 0.015% BND and 0.010% MIT as preservative.
3. ***Control***, 2 vials, 1.0 mL each, ready to use;
   Control values and ranges please refer to vial label or QC-Datasheet.
   * contain 0.03% Proclin 300, 0.015% BND and 0.010% MIT as preservative.
4. ***Enzyme Conjugate***, 1 vial, 26 mL, ready to use;
   Testosterone conjugated to horseradish peroxidase;
   * contain 0.03% Proclin 300, 0.015% BND and 0.010% MIT as preservative.
5. ***Substrate Solution***, 1 vial, 25 mL, ready to use;
   Tetramethylbenzidine (TMB).
6. ***Stop Solution***, 1 vial, 14 mL, ready to use;
   contains 0.5M H_2SO_4.
   Avoid contact with the stop solution. It may cause skin irritations and burns.
7. ***Wash Solution***, 1 vial, 30 ml (40X concentrated);
   see „Preparation of Reagents“.

* BND = 5-bromo-5-nitro-1,3-dioxane
MIT = 2-methyl-2H-isothiazol-3-one

Note: Additional *Standard 0* for sample dilution is available upon request.

Revised 12 Jan. 2008 (Vers. 8.1) **IVD**

Materials required but not provided

1. Calibrated EIA reader adjusted to read at 450 nm
2. Precision pipettes (100 µL and 200 µL)
3. Distilled or Deionized water
4. Timer (60 min. range)
5. Reservoirs (disposable)
6. Test tube or microtube rack in a microplate configuration
7. Semi logarithmic graph paper or software for data reduction

Storage Conditions

When stored at 2°C to 8°C unopened reagents will retain reactivity until expiration date.
Enzyme-Conjugate, Standard Solution, Substrate Solution, Wash Solution and Zero Standard must be stored at 2°C to 8°C.
Microplate wells must be stored at 2°C to 8°C.

Reagent Preparation

Bring all reagents to room temperature before use.

Wash Solution:
Add deionized water to the 40 x concentrated Wash Solution to a final volume of 1200 mL.
The diluted Wash Solution is stable for 2 weeks at room temperature.

SPECIMEN COLLECTION AND PREPARATION

Eating, drinking, chewing gums or brushing teeth should be avoided for 30 minutes before sampling. Otherwise, it is recommended to rinse mouth thoroughly with cold water 5 minutes prior to sampling.

Do not collect samples when oral diseases, inflammation or lesions exist (blood contamination).

If there is visible blood contamination the patient specimen, it should be discarded, rinse the sampling device with water, wait for 10 minutes and take a new sample.

Note: Samples containing sodium azide should not be used in the assay.

Specimen Collection

Saliva samples should be collected only using special saliva sampling devices
(e.g. SALI TUBES 100 REF SLV-4158, available from DRG).
Do not use Salivettes for sampling.

Due to the cyclic secretion pattern of steroid hormones it is important to care for a proper timing of the sampling.
In order to avoid arbitrary results we recommend that 5 samples always be taken within a period of
2 – 3 hours (*multiple sampling*) preferably before a meal.
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.

Revised 12 Jan. 2008 (Vers. 8.1) **IVD**

Specimen Storage and Preparation

The saliva samples may be stored at 2 °C to 8 °C up to one week, and should be frozen at –20 °C for longer periods; repeated thawing and freezing is no problem.

Each sample has to be frozen, thawed, and centrifuged at least once 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 (at 3000 - 2000 x g).
Now the clear colorless supernatant is easy to pipette.

If a set of multiple samples is to be tested, the lab (after at least one freezing, thawing, and centrifugation cycle) has to mix the 5 single samples in a separate sampling device and perform the testing from this mixture.

Specimen Dilution

If in an initial assay, a specimen is found to contain more than the highest standard, 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

Each run must include a standard curve.

1. Secure the desired number of coated strips in the frame holder.
2. Dispense **100 µL** of each Testosterone **Standard** and **Control** into appropriate wells.
3. Dispense **100 µL** of each **sample** into selected wells.
4. Dispense **200 µL** of **Enzyme Conjugate** into each sample and standard well and mix the plate for thoroughly for 10 seconds.
5. Incubate for **60 minutes** at room temperature.
6. Briskly shake out the contents of the wells and rinse the wells **3 times** with diluted Wash Solution (400 µL per well). Strike the inverted wells sharply on absorbent paper towel to remove residual droplets.
7. Add **200 µL** of **Substrate Solution** (4) to each well.
8. Incubate for **30 minutes** at room temperature.
9. Stop the reaction by adding **100 µL** of **Stop Solution** (5) to each well.
10. Determine the absorbance of each well at 450 ± 10 nm.
    It is recommended to read the wells within 10 minutes.

Calculation of Results

1. Calculate the average absorbance values for each set of standards, controls and patient samples.
2. Using linear – log 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 calculation method. Other data reduction functions may give slightly different results.
5. The concentration of the samples can be read 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:
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.

Standard	Absorbance Units
Standard 0 (0 pg/mL)	2.01
Standard 1 (10 pg/mL)	1.89
Standard 2 (50 pg/mL)	1.57
Standard 3 (100 pg/mL)	1.36
Standard 4 (500 pg/mL)	0.69
Standard 5 (1000 pg/mL)	0.43
Standard 6 (5000 pg/mL)	0.13

Revised 12 Jan. 2008 (Vers. 8.1) **IVD**

EXPECTED NORMAL VALUES

In order to determine the normal range of SLV Testosterone, saliva samples from 187 adult male and 188 adult female apparently healthy subjects, ages 21 to 75 years, were collected in the morning and analyzed using the DRG SLV Testosterone ELISA kit. The following range was calculated from this study.

	Men ♂			Women ♀		
Age Group Years	**Range (5 - 95%)**	**Median**	**n**	**Range (5 - 95%)**	**Median**	**n**
21 - 30	47.2 –136.2	92.8	42	7.9 – 50.4	20.8	40
31 - 40	46.8 –106.8	73.6	37	<7.0 – 44.8	17.1	40
41 - 50	36.5 –82.7	58.8	34	<7.0 – 39.4	18.3	38
51 - 60	19.1 – 89.0	44.5	36	<7.0 – 29.8	19.2	38
61 - 75	12.2 – 68.6	38.9	38	<7.0 – 29.3	16.0	32

The results alone should not be the only reason for therapy. The results should be correlated to other clinical observations and diagnostic tests. Since testosterone levels show diurnal cycles, we recommend that the samples be obtained the same hour each day. Furthermore, we recommend that each laboratory determine its own range for the population tested.

Quality Control

Good laboratory practice requires that controls be run with each calibration 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 state and federal 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 on 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 analysing control values and trends. If the results of the assay do not fit to the established acceptable ranges of control materials patient 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.
After checking the above mentioned items without finding any error contact your distributor or DRG directly.

PERFORMANCE CHARACTERISTICS

Sensitivity

The lowest analytical detectable level of testosterone that can be distinguished from the Zero Standard is 1.9 pg/mL at the 95 % confidence limit.

The lowest functional sensitivity of 7.1 pg/mL at the 95% confidence limit was obtained.

Specificity

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	0.80
Androstenedione	0.90
11β-hydroxysterone	3.30
17α-methyltestosterone	0.10
19-Nortestosterone	3.30
Epitestosterone	0.10
Estradiol	0.10
Progesterone	< 0.10
Cortisol	< 0.10
Estrone	< 0.10
Danazol	< 0.10

Reproducibility

Intra-Assay

The intra-assay variation was determined by 20 replicate measurements of 5 saliva samples within one run. The within-assay variability is shown below:

Mean (pg/mL)	144.0	256.2	81.3	35.4	12.9
SD	9.0	17.6	5.5	2.5	1.8
CV (%)	6.2	6.9	6.8	7.1	13.8
n =	20	20	20	20	20

Inter-Assay

The inter-assay (between-run) variation was determined by duplicate measurements of 5 saliva samples over 10 days.

Mean (pg/mL)	823.1	87.6	118.8	112.1	33.6
SD	45.3	6.9	8.9	8.6	3.2
CV (%)	5.5	7.8	7.5	7.7	9.6
n =	20	20	20	20	20

Inter-Lot

The Inter-Lot (between-lot) variation was determined by triplicate measurements of five saliva samples in three different kit lots. The between lot variability is shown below:

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Mean (pg/mL)	64.5	352.9	517.7	44.0	116.5
SD (pg/mL)	3.8	13.4	15.0	1.5	5.0
CV (%)	5.9	3.8	2.9	3.5	4.3
n =	9	9	9	9	9

Recovery

Recovery of the DRG ELISA was determined by adding increasing amounts of the analyte to six different saliva samples containing different amounts of endogenous analyte. Each sample (native and spiked) was assayed and analyte concentrations of the samples were calculated from the standard curve. The percentage recoveries were determined by comparing expected and measured values of the samples

Sample	Measured (pg/mL)	Expected (pg/mL)	Recovery (%)
1	8.4	-	-
	2396.9	2508.4	95.6
	517.2	508.4	101.7
	271.1	258.4	104.9
	56.0	58.4	95.82
2	46.2	-	-
	2474.7	2546.2	97.2
	564.4	546.2	103.3
	309.0	296.2	104.3
	88.9	96.2	92.4
3	122.1	-	-
	2602.4	2622.1	99.3
	591.2	622.1	95.0
	352.6	372.1	94.8
	166.4	172.1	96.7

Sample	Measured (pg/mL)	Expected (pg/mL)	Recovery (%)
4	210	-	-
	2650	2710	97.8
	700	710	98.6
	240	240	100.0
5	1250	-	-
	3700	3750	98.7
	1680	1750	96.0
	1250	1280	97.7
6	2090	-	-
	4550	4590	99.1
	2650	2590	102.3
	2130	2120	100.5

Linearity

Six saliva samples containing different amounts of analyte were serially diluted with zero standard and assayed with the DRG ELISA. Three native samples were serially diluted, and 3 samples were spiked with testosterone and then serially diluted up to 1:128. The percentage recovery was calculated by comparing the expected and measured values for testosterone. An assay linearity of 7.1 – 4500 pg/mL has been identified as the usable range. Samples above this range must be diluted and re-run.

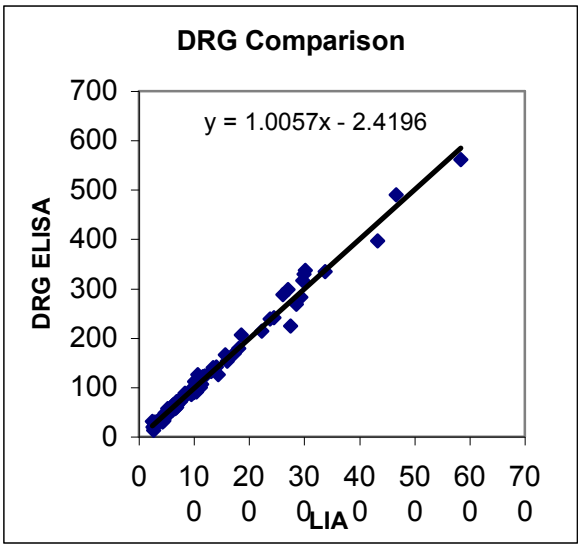
Revised 12 Jan. 2008 (Vers. 8.1) **IVD**

		Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
Concentration (pg/mL)		4312	1838	440	8000	4500	5500
Average % Recovery		97.5	99.6	98.9	100.3	100.8	100.7
Range of Recovery %	**from**	96.3	98.4	93.6	93.6	93.7	94.1
	to	98.7	101.0	106.7	106.7	107.0	107.8

Comparison Studies

A study was performed that evaluated saliva samples from 99 male and female subjects ages 20 to 70 years. The saliva samples were run in duplicate on the DRG test and a commercially available LIA method to determine the concentration of free Testosterone in the samples. A correlation of 0.904 and regression formula of y = 0.9251x - 7.4369 was obtained versus this method.

Another study was performed to further evaluate the substantial equivalence of the DRG SLV Testosterone to the LIA saliva test. The concentration of testosterone in 81 additional saliva samples collected from 40 - 65 year old men and women was determined using DRG SLV testosterone kit and the other method. From this study an $R^2 = 0.9866$ was obtained with the following regression.



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.
Blood contamination of more than 0.16 % in saliva samples will affect results, and usually can be seen by eye.
Concentrations of Sodium Azide ≥ 0.02 % interferes in this assay and may lead to false results.

Revised 12 Jan. 2008 (Vers. 8.1) **IVD**

REFERENCES

1. Mulhall JP, et al. (2004); Effect of testosterone supplementation on sexual function in hypogonadal men with erectile dysfunction, Urology 63, 348-352
2. Grinspoon s., et al. (2000); Effects of hypogonadism and testosterone administration on depression indices in HIV-infected men, J Clin. Endocrinol Metab. 85, 60-65
3. Leder BZ, et al. (2004): Effect of aromatase inhibition in elderly men with low or borderline-low serum testosterone levels, J Clin Endocrinol Metab. 89, 1174-1180
4. Bunch T., et al. (2002): Pituitary radiographic abnormalities and clinical correlates of hypogonadism in elderly males presenting with erectile dysfunction, Aging Male. 5, 38-46
5. Ruutiainen K., etal. (1987): Salivary testosterone in hirsutism: correlations with serum testosterone and the degree of hair growth, J Clin Endocrinol Metab. 64, 1015-1020
6. Cserepes E., et al. (2002): Ovarian steroid cell tumor and a contralateral Ovarian thecoma in a postmenopausal woman with severe hyperandrogenism, Gynecol Endocrinol. 16, 213-216
7. Baldwin L. J., et al; (2004): Virilisation in a case of transitional cell carcinoma of the ovary, J Clin Pathol. 57, 1331-1332
8. Blumberg J., et al. (1991): Testicular tumors in congenital adrenal hyperplasia: steroid measurements from adrenal and spermatic veins, J Clin Endocrinol Metab. 73, 1129-1133