



Diagnostics Biochem Canada Inc.

Manufacturer of In Vitro Diagnostic Test Kits Since 1973

**ELISA Kit...The EiAsy™ Way
TESTOSTERONE SALIVA**

Cat. No.: CAN-TE-300

Version: 4.0

Effective: November 14, 2006

INTENDED USE

For the quantitative determination of Testosterone by enzyme immunoassay in human saliva.

For *in vitro* diagnostic use only.

PRINCIPLE OF THE TEST

The principle of the following enzyme immunoassay test follows a two-step competitive binding scenario. Competition occurs between an unlabeled antigen (present in standards, control and patient samples) and a biotin-labelled antigen (conjugate) for a limited number of antibody binding sites on the microwell plate. In the second step, the streptavidin-horseradish peroxidase conjugate binds to any bound biotinylated testosterone. The washing and decanting procedures remove unbound materials. After the last washing step, the enzyme substrate is added. The enzymatic reaction is terminated by addition of the stopping solution. The absorbance is measured on a microtiter plate reader. The intensity of the colour formed is inversely proportional to the concentration of testosterone in the sample. A set of standards is used to plot a standard curve from which the amount of testosterone in patient samples and controls can be directly read.

CLINICAL APPLICATIONS

Testosterone is a C-19 steroid secreted from the testis and the adrenal cortex in men and from the adrenal cortex and ovary in women. Testosterone is also produced by peripheral tissues from androstenedione, which is of little physiological significance in men, however in women about half of circulating testosterone is derived from this origin. The action of testosterone is both androgenic or anabolic. Testosterone measurements are used mainly for clinical evaluation of hypogonadism in males and hyperandrogenic states in females.

Most of the circulating testosterone is bound to three proteins: sex hormone binding globulin (44-78%), albumin (20-54%) and cortisol binding globulin (small amount). Only about 2-3% of the total circulating testosterone remains unbound or in the free form. Only the free portion (or the non-SHBG bound fraction) of the circulating testosterone is thought to be available to tissues where it exerts its biological actions.

The salivary hormone assays are advocated for their noninvasive, easy sample collection method. Salivary testosterone is of great clinical value for it represents a filtered fraction of plasma testosterone and is independent of flow rate. Many studies have suggested that salivary testosterone correlates well with either free or non-SHBG bound testosterone.

PROCEDURAL CAUTIONS AND WARNINGS

1. Users should have a thorough understanding of this protocol for the successful use of this kit. Reliable performance will only be attained by strict and careful adherence to the instructions provided.

2. Control materials should be included in every run at a high and low level for assessing the reliability of results.

- When the use of water is specified for dilution or reconstitution, use deionized or distilled water.
- In order to reduce exposure to potentially harmful substances, gloves should be worn when handling kit reagents and human specimens.
- All kit reagents and specimens should be brought to room temperature and mixed gently but thoroughly before use. Avoid repeated freezing and thawing of reagents and specimens.
- A calibrator curve must be established for every run.
- The control should be included in every run and fall within established confidence limits.
- Improper procedural techniques, imprecise pipetting, incomplete washing as well as improper reagent storage may be indicated when assay values for the control do not reflect established ranges.
- When reading the microplate, the presence of bubbles in the microwells will affect the optical densities (ODs). Carefully remove any bubbles before performing the reading step.
- The substrate solution (TMB) is sensitive to light and should remain colourless if properly stored. Instability or contamination may be indicated by the development of a blue colour, in which case it should not be used.
- When dispensing the substrate and stopping solution, do not use pipettes in which these liquids will come into contact with any metal parts.
- To prevent contamination of reagents, use a new disposable pipette tip for dispensing each reagent, sample, standard and control.
- Do not mix various lot numbers of kit components within a test and do not use any component beyond the expiration date printed on the label.
- Kit reagents must be regarded as hazardous waste and disposed of according to national regulations.

LIMITATIONS

- All the reagents within the kit are calibrated for the direct determination of testosterone in human saliva. The kit is not calibrated for the determination of testosterone in serum, plasma or other specimens of human or animal origin.
- Any samples or control sera containing azide or thimerosal are not compatible with this kit, as they may lead to false results.
- Only calibrator A may be used to dilute any high saliva samples. The use of any other reagent may lead to false results.
- The results obtained with this kit should never be used as the sole basis for a clinical diagnosis. For example, the occurrence of heterophilic antibodies in patients regularly exposed to animals or animal products has the potential of causing interferences in immunological tests. Consequently, the clinical diagnosis should include all aspects of a patient's background including the frequency of exposure to animals/products if false results are suspected.

**SAFETY CAUTIONS AND WARNINGS
POTENTIAL BIOHAZARDOUS MATERIAL**

Human serum that may be used in the preparation of the standards and control has been tested and found to be non-reactive for Hepatitis B surface antigen and has also been tested for the presence of antibodies to HCV and Human Immunodeficiency Virus (HIV) and found to be negative. However no test method can offer complete assurance that HIV, HCV and Hepatitis B virus or any infectious agents are absent. The reagents should be considered a potential biohazard and handled with the same precautions as applied to any blood specimen.

CHEMICAL HAZARDS

Avoid contact with reagents containing TMB, hydrogen peroxide and sulfuric acid. If contacted with any of these reagents, wash with plenty of water. TMB is a suspected carcinogen.

SPECIMEN COLLECTION AND STORAGE

Approximately 1 ml of saliva is required per duplicate determination. Collect 2-3 ml of saliva into a clean glass tube without force or inducement and before eating, drinking or brushing the teeth. Simply rinse the mouth with water before collection. Do not use blood-contaminated specimens. Store samples at 4°C for up to 24 hours or at -10°C or lower if the analyses are to be done at a later date. Consider all human specimens as possible biohazardous materials and take appropriate precautions when handling.

SPECIMEN PRETREATMENT

- Specimen samples are to be centrifuged. The supernatants are to be transferred into clean tubes.
- The tubes containing the supernatant are to be placed in a waterbath and heated at 60-70°C for 1 hour.
- Allow heated samples to reach room temperature before assaying.

REAGENTS AND EQUIPMENT NEEDED BUT NOT PROVIDED

- Precision pipettes to dispense 50, 100, 150 and 300 µl
- Disposable pipette tips
- Distilled or deionized water
- Plate shaker
- Benchtop centrifuge
- Microwell plate reader with a filter set at 450nm and an upper OD limit of 3.0 or greater* (see assay procedure step 13).
- Waterbath

REAGENTS PROVIDED

1. Rabbit Anti-Testosterone Antibody Coated Microwell Plate-Break Apart Wells - Ready To Use.

Contents: One 96 well (12x8) polyclonal antibody-coated microwell plate in a resealable pouch with desiccant.
Storage: Refrigerate at 2-8°C
Stability: 12 months or as indicated on label.

2. Testosterone-Biotin Conjugate (Biotin Conjugate) Concentrate - Requires Preparation.

Contents: Testosterone-Biotin conjugate in a protein-based buffer with a non-mercury preservative.
Volume: 300 µl/vial
Storage: Refrigerate at 2-8°C
Stability: 12 months or as indicated on label.
Preparation: Dilute 1:50 in biotin conjugate assay buffer before use. Discard any unused solution.

3. Streptavidin-Horse Radish Peroxidase (HRP) Conjugate Concentrate (HRP Conjugate) - Requires Preparation.

Contents: Streptavidin-HRP conjugate in a protein-based buffer with a non-mercury preservative.
Volume: 400 µl/vial
Storage: Refrigerate at 2-8°C
Stability: 12 months or as indicated on label.
Preparation: Dilute 1:50 in HRP conjugate assay buffer before use. Discard any unused solution.

4. Testosterone Saliva Calibrators - Ready To Use.

Contents: Six vials containing testosterone in a protein-based buffer with a non-mercury preservative. Prepared by spiking buffer with a defined quantity of testosterone.

*Listed below are approximate concentrations, please refer to vial labels for exact concentrations.

Calibrator	Concentration	Volume/Vial
Calibrator A	0 pg/ml	4.0 ml
Calibrator B	2 pg/ml	1.0 ml
Calibrator C	10 pg/ml	1.0 ml
Calibrator D	50 pg/ml	1.0 ml
Calibrator E	200 pg/ml	1.0 ml
Calibrator F	800 pg/ml	1.0 ml

Storage: Refrigerate at 2-8°C

Stability: 12 months in unopened vials or as indicated on label. Once opened, the standards should be used within 14 days or aliquoted and stored frozen. Avoid multiple freezing and thawing cycles.

5. Control - Ready To Use.

Contents: One vial containing testosterone in a protein-based buffer with a non-mercury preservative. Prepared by spiking buffer with a defined quantity of testosterone. Refer to vial label for expected value and acceptable range.

Volume: 1.0 ml/vial

Storage: Refrigerate at 2-8°C

Stability: 12 months in unopened vial or as indicated on label. Once opened, the control should be used within 14 days or aliquoted and stored frozen. Avoid multiple freezing and thawing cycles.

6. Biotin Conjugate Assay Buffer - Ready To Use.

Contents: One vial containing a protein-based buffer with a non-mercury preservative.

Volume: 13 ml/vial

Storage: Refrigerate at 2-8°C

Stability: 12 months or as indicated on label.

7. HRP Conjugate Assay Buffer - Ready To Use.

Contents: One vial containing a protein-based buffer with a non-mercury preservative.

Volume: 20 ml/vial

Storage: Refrigerate at 2-8°C

Stability: 12 months or as indicated on label.

8. Wash Buffer Concentrate - Requires Preparation.

Contents: One bottle containing buffer with a non-ionic detergent and a non-mercury preservative.

Volume: 50 ml/bottle

Storage: Refrigerate at 2-8°C

Stability: 12 months or as indicated on label.

Preparation: Dilute 1:10 in distilled or deionized water before use. If the whole plate is to be used dilute 50 ml of the wash buffer concentrate in 450 ml of water.

9. TMB Substrate - Ready To Use.

Contents: One bottle containing tetramethylbenzidine and hydrogen peroxide in a non-DMF or DMSO containing buffer.

Volume: 16 ml/bottle

Storage: Refrigerate at 2-8°C

Stability: 12 months or as indicated on label.

10. Stopping Solution - Ready To Use.

Contents: One vial containing 1M sulfuric acid.

Volume: 6 ml/vial

Storage: Refrigerate at 2-8°C

Stability: 12 months or as indicated on label.

ASSAY PROCEDURE

Specimen Pretreatment:

Centrifugation and Heating at 60-70°C for 1 Hour

All reagents must reach room temperature before use. Calibrators, controls and specimen samples should be assayed in duplicate. Once the procedure has been started, all steps should be completed without interruption.

1. Prepare working solutions of the testosterone-biotin conjugate, streptavidin-HRP conjugate and wash buffer.
2. Remove the required number of microwell strips. Reseal the bag and return any unused strips to the refrigerator.
3. Pipette 100 µl of each calibrator, control and specimen sample into correspondingly labelled wells in duplicate.
4. Pipette 100 µl of the testosterone-biotin conjugate working solution into each well (We recommend using a multichannel pipette).
5. Incubate on a plate shaker (approximately 200 rpm) for 1 hour at room temperature.
6. Wash the wells 5 times with 300 µl of diluted wash buffer per well and tap the plate firmly against absorbent paper to ensure that it is dry (The use of a washer is recommended).
7. Pipette 150 µl of the streptavidin-HRP conjugate working solution into each well (We recommend using a multichannel pipette).
8. Incubate on a plate shaker (approximately 200 rpm) for 30 minutes at room temperature.
9. Wash the wells again in the same manner as step 6.
10. Pipette 150 µl of TMB substrate into each well at timed intervals.
11. Incubate on a plate shaker for 10-15 minutes at room temperature (or until calibrator A attains dark blue colour for desired OD).
12. Pipette 50 µl of stopping solution into each well at the same timed intervals as in step 10.
13. Read the plate on a microwell plate reader at 450nm within 20 minutes after addition of the stopping solution.

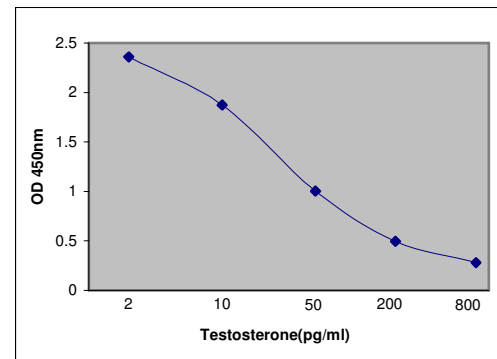
* If the OD exceeds the upper limit of detection or if a 450nm filter is unavailable, a 405 or 415nm filter may be substituted. The optical densities will be lower, however, this will not affect the results of patient/control samples.

CALCULATIONS

1. Calculate the mean optical density of each calibrator duplicate.
2. Draw a calibrator curve on semi-log paper with the mean optical densities on the Y-axis and the calibrator concentrations on the X-axis. If immunoassay software is being used, a 4-parameter curve is recommended.
3. Calculate the mean optical density of each unknown duplicate.
4. Read the values of the unknowns directly off the calibrator curve.
5. If a sample reads more than 800 pg/ml then dilute it with calibrator A at a dilution of no more than 1:8. The result obtained should be multiplied by the dilution factor.

TYPICAL TABULATED DATA

Calibrator	OD 1	OD 2	Mean OD	Value (pg/ml)
A	2.739	2.591	2.665	0
B	2.385	2.338	2.362	2
C	1.857	1.887	1.872	10
D	0.987	1.027	1.007	50
E	0.497	0.495	0.496	200
F	0.291	0.273	0.282	800



Unknown	1.086	1.077	1.082	43.7
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TYPICAL CALIBRATOR CURVE

Sample curve only. Do not use to calculate results.

PERFORMANCE CHARACTERISTICS**SENSITIVITY**

The lower detection limit is calculated from the standard curve by determining the resulting concentration of the mean OD of Calibrator A (based on 10 replicate analyses) minus 2 SD. Therefore, the sensitivity of the dbc Testosterone Saliva ELISA kit is **1.0 pg/ml**.

SPECIFICITY (CROSS REACTIVITY)

The following compounds were tested for cross-reactivity with the Testosterone ELISA kit with testosterone cross-reacting at 100%.

Steroid	%Cross Reactivity
Testosterone	100
5α-DHT	5.2
Androstenedione	1.4
Androstanediol	0.8
Progesterone	0.5
Androsterone	0.1

The following steroids were tested but cross-reacted at less than 0.1%: Aldosterone, Andrenosterone, Cholesterol,

Corticosterone, Dehydroepiandrosterone, Dehydroepiandrosterone Sulfate, Epiandrosterone, 17β-Estradiol, Estriol and Pregnenolone.

INTRA-ASSAY PRECISION

Three samples were assayed ten times each on the same calibrator curve. The results (in pg/ml) are tabulated below:

Sample	Mean	SD	CV%
1	14.05	1.00	7.1
2	38.19	1.30	3.4
3	122.81	8.19	6.7

INTER-ASSAY PRECISION

Three samples were assayed ten times over a period of four weeks. The results (in pg/ml) are tabulated below:

Sample	Mean	SD	CV%
1	11.03	1.08	9.8
2	47.24	3.32	7.0
3	122.79	11.23	9.1

RECOVERY

Spiked samples were prepared by adding defined amounts of testosterone to 3 patient saliva samples. The results (in pg/ml) are tabulated below:

Sample	Obs.Result	Exp.Result	Recovery%
1 Unspiked	5.21	-	-
+ 50(5:1)	15.89	14.2	114
+200(5:1)	50.82	44.0	116
+800(5:1)	154.48	164.0	94
2 Unspiked	41.80	-	-
+ 50(5:1)	34.86	36.0	97
+200(5:1)	57.39	66.4	86
+800(5:1)	165.73	186.0	89
3 Unspiked	52.57	-	-
+ 50(5:1)	53.67	52.08	97
+200(5:1)	85.77	83.00	97
+800(5:1)	179.00	203.00	89

LINEARITY

Three patient saliva samples were diluted with calibrator A. The results (in pg/ml) are tabulated below:

Sample	Obs.Result	Exp.Result	Recovery%
1	154	-	-
1:2	82.44	77.24	107
1:4	39.38	38.62	102
1:8	23.14	19.31	120
2	165.73	-	-
1:2	88.74	82.87	99
1:4	50.47	41.43	122
1:8	16.95	20.72	82
3	188.75	-	-
1:2	104.45	94.38	111
1:4	49.24	47.19	104
1:8	20.31	23.59	86

EXPECTED NORMAL VALUES

As for all clinical assays each laboratory should collect data and establish their own range of expected normal values.

Group	N	Range (pg/ml)
Males	40	38-120
Females	41	5 – 32

EXTRACTION VS. NON-EXTRACTION COMPARITIVE STUDY

The dbc Testosterone Saliva ELISA method was validated by the following comparative study between:

1. Prior extraction of saliva samples with diethyl ether
 2. Prior heating of saliva samples for 1 hour at 60-70°C
- The results (in pg/ml) are tabulated below:

Sample	Extracted	Heated
1	37	44
2	57	52
3	35	35
4	42	36
5	44	41
6	35	32
7	37	33
8	41	35
9	49	51
10	45	53
11	37	19
12	22	25
13	11	9.59
14	55	52.17
15	33	33.08

The data shows as a strong agreement between the two methods, with a correlation of r=0.8747

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