EAGLE BIOSCIENCES

Testosterone Saliva LIA Assay Kit

Catalog Number:
TST32-L01 (1 x 96 wells)
For Research Use Only. Not for use in diagnostic procedures.
v. 1.0

EAGLE BIOSCIENCES, INC.
20A Northwest Blvd., Suite 112, Nashua, NH 03063
Phone: 617-419-2019 Fax: 617-419-1110
WWW.EAGLEBIO.COM

INTENDED USE

The Eagle Biosciences Testosterone Saliva LIA Assay Kit is for the direct quantitative determination of testosterone in human saliva by a chemiluminescence immunoassay (LIA). The Eagle Biosciences Testosterone Saliva LIA assay kit is for research use only and not to be used in diagnostic procedures.

INTRODUCTION

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 and 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.

PRINCIPLE OF THE ASSAY

The principle of the following chemiluminescence immunoassay (LIA) test follows a two-step competitive binding scenario. Competition occurs between an unlabelled antigen (present in standards, controls and patient samples) and a biotin-labelled antigen (conjugate) for a limited number of antibody binding sites on the microplate. After washing the streptavidin-horseradish peroxidase conjugate is incubated and bound to any bound biotinylated testosterone. The washing and decanting procedures remove unbound materials. After the second washing step, the luminescence substrate solution is added. The relative luminescence units (RLUs) are measured on a microtiter plate luminometer. The RLU values are inversely proportional to the concentration of testosterone in the sample. A set of calibrators is used to plot a standard curve from which the amount of testosterone in patient samples and controls can be directly read.

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 or serum pools should be included in every run at a high and low level for assessing the reliability of results.
3. When the use of water is specified for dilution or reconstitution, use deionized or distilled water.
4. In order to reduce exposure to potentially harmful substances, gloves should be worn when handling kit reagents and human specimens.
5. 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.
6. A calibrator curve must be established for every run.
7. The kit controls should be included in every run and fall within established confidence limits.
8. Improper procedural techniques, imprecise pipetting, incomplete washing as well as improper reagent storage may be indicated when assay values for the controls do not reflect

established ranges. The performance of this assay is markedly influenced by the correct execution of the washing procedure!
9. The luminescence substrate solutions (A and B) are sensitive to light and should be stored in the original dark bottle away from direct sunlight.
10. When dispensing the substrate, do not use pipettes in which these liquids will come into contact with any metal parts.
11. To prevent contamination of reagents, use a new disposable pipette tip for dispensing each reagent, sample, standard and control.
12. 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.
13. Kit reagents must be regarded as hazardous waste and disposed of according to national regulations.

LIMITATIONS
1. All the reagents within the kit are calibrated for the 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.
2. Do not use grossly hemolyzed, grossly lipemic, icteric or improperly stored serum.
3. Only calibrator A may be used to dilute any high saliva samples. The use of any other reagent may lead to false results.
4. This kit is intended for research use only and should not be used in diagnostic procedures.

SAFETY CAUTIONS AND WARNINGS
POTENTIAL BIOHAZARDOUS MATERIAL
Human fluids that may be used in the preparation of the standards and controls has been tested and found to be nonreactive 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. No test method however, 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 direct contact with reagents. In case of contact, wash with plenty of water.

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 and wait a few minutes to start. Do not use blood-contaminated specimens.

SPECIMEN PRETREATMENT
This assay is a direct system; no specimen pretreatment is necessary.

REAGENTS AND EQUIPMENT NEEDED BUT NOT PROVIDED
1. Precision pipettes to dispense 100, 150 and 300 µL
2. Disposable pipette tips
3. Distilled or deionized water
4. Plate shaker
5. Microplate luminometer
6. Water bath

REAGENTS PROVIDED AND PREPARATION

1. Rabbit Anti-Testosterone Antibody-Coated Break-Apart Well Microplate — Ready To Use
   - Contents: One 96-well (12x8) polyclonal antibody-coated microplate in a resealable pouch with desiccant.
   - Storage: Refrigerate at 2–8°C
   - Stability: 12 months or as indicated on label.

2. Testosterone-Biotin Conjugate Concentrate – Requires Preparation x100
   - Contents: Testosterone-biotin conjugate in a protein-based buffer with a non-mercury preservative.
   - Volume: 0.2 mL/vial
   - Storage: Refrigerate at 2–8°C
   - Stability: 12 months or as indicated on label.

   Preparation of conjugate working solution: Dilute biotin conjugate concentrate 1:100 in biotin conjugate buffer before use (example: 20 µL of biotin conjugate concentrate in 2 mL of biotin conjugate buffer). If the whole plate is to be used dilute 120 µL of biotin conjugate concentrate in 12 mL of biotin conjugate buffer. Discard any that is left over.

3. Streptavidin- Horseradish Peroxidase (HRP) Conjugate Concentrate — Requires Preparation X100
   - Contents: Streptavidin-HRP conjugate in a protein-based buffer with a non-mercury preservative.
   - Volume: 0.3 mL/vial
   - Storage: Refrigerate at 2–8°C
   - Stability: 12 months or as indicated on label.

   Preparation of conjugate working solution: Dilute HRP conjugate concentrate 1:100 in HRP conjugate buffer before use (example: 20 µL of conjugate concentrate in 2 mL of assay buffer). If the whole plate is to be used dilute 180 µL of HRP conjugate concentrate in 18 mL of HRP conjugate buffer. Discard any that is left over.

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 bottle 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. Controls — Ready To Use
   - Contents: Two vials containing testosterone in a protein-based buffer with a non-mercury preservative. Prepared by spiking buffer with a defined quantity of testosterone. Refer to vials label for expected value and acceptable range.
   - Volume: 1 mL/vial
   - 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.

6. Biotin Conjugate Buffer – Ready To Use
   - Contents: One bottle containing a protein-based buffer with a non-mercury preservative
   - Volume: 13 mL/bottle
   - Storage: Refrigerate at 2–8°C
   - Stability: 12 months or as indicated on label.

7. HRP Conjugate Buffer
   - Conjugate: One bottle containing a protein-based buffer with a non-mercury preservative.
   - Volume: 20 mL/bottle
   - Storage: Refrigerate at 2–8°C
   - Stability: 12 months or as indicated on label.

8. Wash Buffer Concentrate – Requires Preparation X10
   - 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 one whole plate is to be used dilute 50 mL of the wash buffer concentrate in 450 mL of water.

9. LIA Substrate Reagent A – Requires Preparation
   - Contents: One vial containing luminol plus enhancer.
   - Volume: 0.8 mL/vial
   - Storage: Refrigerate at 2–8°C
   - Stability: 12 months or as indicated on label.
   - Preparation: See preparation of LIA working substrate solution.

10. LIA Substrate Reagent B – Requires Preparation
    - Contents: One vial containing stabilized peroxide solution.
    - Volume: 1.6 mL/vial
    - Storage: Refrigerate at 2–8°C
    - Stability: 12 months or as indicated on label.
    - Preparation: See preparation of LIA working substrate solution.

11. LIA Substrate Reagent C – Requires Preparation

Contents:	One bottle containing buffer with a non-mercury preservative.
Volume:	15 mL/bottle
Storage:	Refrigerate at 2–8°C
Stability:	12 months or as indicated on label.
Preparation:	See preparation of LIA working substrate solution.

PREPARATION OF LIA WORKING SUBSTRATE SOLUTION

In a clean container mix 1 part of LIA substrate reagent A with 2 parts of LIA substrate reagent B and 20 parts of LIA substrate reagent C. This gives the ready to use substrate solution. If the whole plate is to be used, prepare working substrate solution as follows: Combine 0.7 mL of LIA substrate reagent A with 1.4 mL of LIA substrate reagent B and 14 mL of LIA substrate reagent C. It is suggested to wait at least 2 minutes prior to use after preparation of the working substrate solution. The working substrate solution is stable for up to 2 hours at room temperature. Discard the leftovers.

ASSAY PROCEDURE

Important Notes:

- All reagents must reach room temperature before use.
- Once the procedure has been started, all steps should be completed without interruption to ensure equal elapsed time for each pipetting step.
- The washing procedure influences the precision markedly; it is essential to ensure the washing is effective and thorough.

1. Prepare working solutions of both conjugates, wash buffer and LIA substrate (refer to reagents provided and preparation section).
2. Remove the required number of well strips. Reseal the bag and return any unused strips to the refrigerator.
3. Pipette 100 µL of each calibrator, controls and pretreated specimen sample (refer to specimen pretreatment section) 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. Cover the plate and incubate for 1 hour on a plate shaker (approximately 200 rpm) 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. Cover the plate and incubate for 30 minutes on a plate shaker (approximately 200 rpm) at room temperature.
9. Wash the wells again in the same manner as step 6.
10. Pipette 100 µL of LIA working substrate solution into each well. (We recommend using a multichannel pipette.)
11. Measure the RLU/second in each well on a microplate luminometer within 10–30 minutes after addition of the substrate.

CALCULATIONS

1. Calculate the mean RLU of each calibrator duplicate.
2. Draw a calibrator curve on semi-log paper with the mean RLUs 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 RLU 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.

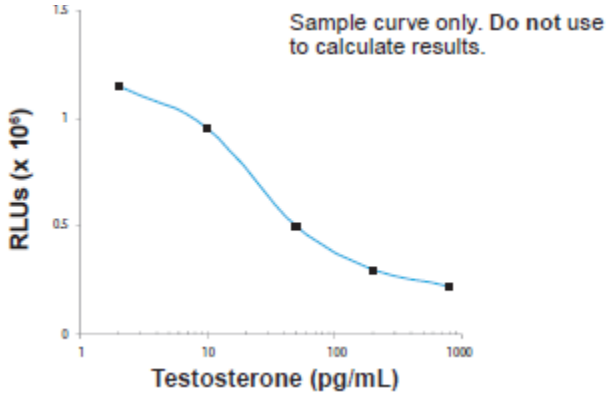
TYPICAL TABULATED DATA**

Sample data only. Do not use to calculate results.

Calibrator	RLU 1	RLU 2	Mean RLU	RLU/RLU_{MAX} (%)
A, 0 pg/mL	2083570	2021850	2052710	100
B, 2 pg/mL	1880710	1818140	1849425	90
C, 10 pg/mL	1103890	1098340	1101115	54
D, 50 pg/mL	336670	345240	340955	17
E, 200 pg/mL	117500	106900	112200	5
F, 800 pg/mL	45430	45580	45505	2

** It is recommended to use the RLU/RLU_{MAX} values for comparative purposes since luminometers vary considerably between manufacturers. Results from different luminometers will show quite different RLU values, however, the RLU/RLU_{MAX} values remain consistent.

TYPICAL CALIBRATOR CURVE



PERFORMANCE CHARACTERISTICS

SENSITIVITY

The lower detection limit is calculated from the standard curve by determining the resulting concentration of the mean RLU of calibrator A (based on 10 replicate analyses) minus 2 SD. Therefore, the sensitivity of the DBC testosterone saliva LIA kit is **1.0 pg/mL.**

SPECIFICITY (CROSS-REACTIVITY)

The following compounds were tested for cross-reactivity with the testosterone saliva LIA 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	12.23	0.91	7.4
2	23.89	1.65	6.9
3	56.14	4.38	7.8

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	1.30	11.8
2	25.34	1.77	7.0
3	57.89	5.85	10.1

RECOVERY

Spiked samples were prepared by adding defined amounts of testosterone to three 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

EXTRACTION Vs. NON-EXTRACTION COMPARATIVE STUDY

The testosterone saliva LIA 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

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	30-120
Females	41	3-22

REFERENCES

1. Johnson SG, et al. Clin Chim Acta. 1987; 163(3):309–18.
2. Carter GD, et al. Ann Clin Biochem. 1983; 20(Pt 5):262–3.
3. Baxendale PM, et al. Clin Endocrinol (Oxf). 1982; 16(6): 595–603.
4. Luisi M, et al. J Steroid Biochem. 1980; 12:513–6.
5. Turkes A, et al. Steroids. 1979; 33(3):347–59.
6. Gaskell SJ, et al. Steroids. 1980; 36(2):219–28.
7. Cheng RW, et al. Clin Chem. 1986; 32(7):1411.
8. Wang C, et al. J Clin Endocrinol Metab. 1981; 53(5):1021–4.

Warranty Information

Eagle Biosciences, Inc. warrants its Product(s) to operate or perform substantially in conformance with its specifications, as set forth in the accompanying package insert. This warranty is expressly limited to the refund of the price of any defective Product or the replacement of any defective Product with new Product. This warranty applies only when the Buyer gives written notice to the Eagle Biosciences within the expiration period of the Product(s) by the Buyer. In addition, Eagle Biosciences has no obligation to replace Product(s) as result of a) Buyer negligence, fault, or misuse, b) improper use, c) improper storage and handling, d) intentional damage, or e) event of force majeure, acts of God, or accident.

Eagle Biosciences makes no warranties, either expressed or implied, except as provided herein, including without limitation thereof, warranties as to marketability, merchantability, fitness for a particular purpose or use, or non-infringement of any intellectual property rights. In no event shall the company be liable for any indirect, incidental, or consequential damages of any nature, or losses or expenses resulting from any defective product or the use of any product. Product(s) may not be resold, modified, or altered for resale without prior written approval from Eagle Biosciences, Inc.

For further information about this kit, its application or the procedures in this kit, please contact the Technical Service Team at Eagle Biosciences, Inc. at info@eaglebio.com or at 866-411-8023.